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The Impact of Soluble Microbial Products on Trace Organic Contaminant Degradation

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The Impact of Soluble Microbial Products on Trace Organic Contaminant Degradation

by

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Dedication

To my parents, for the immense support since day one.

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Abstract

The Impact of Soluble Microbial Products on Trace Organic Contaminant Degradation

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With the increasing popularity of biological filtration for drinking water treatment, a better understanding is needed of the biological removal mechanisms of trace organic contaminants (TrOC). Microorganisms in drinking-water biofilters excrete soluble microbial products (SMP), which provide an additional carbon source to the heterotrophic microbial community. The primary objective of this research was to investigate if heterotrophic microorganisms that are acclimated to complex SMP produced by nitrifying microorganisms will transform TrOC at an increased rate as compared to heterotrophic microorganisms that are acclimated to simple carbon sources.

Batch experiments were conducted with a mixed heterotrophic culture and a pure culture, *Pseudomonas aeruginosa*, fed either a simple organic (acetate) or SMP. After the cultures were acclimated to these carbon sources, they were spiked with two TrOC, 2-methylisoborneol (2-MIB) and geosmin. In addition, two controls were run: an azide control to account for sorption to biomass and a no-biomass control to account for sorption to glassware and volatilization. The removal of 2-MIB and geosmin in the cultures was monitored over time.

These batch experiments demonstrated no substantial advantage in 2-MIB or geosmin biodegradation due to heterotrophic acclimation to this particular SMP as compared to acclimation to acetate. This result might have been influenced by the low specific ultraviolet absorbance (SUVA) of the SMP in this study (0.083 L/mg-min),

which suggests a low degree of aromaticity in the mixture. However, the data suggest that these particular SMP instigate co-metabolic geosmin removal. To better understand the effect of nitrifier SMP on TrOC removal in drinking-water biofilters, future experiments should be conducted with several well-characterized, complex organic mixtures that have higher SUVA values than that of the SMP in this study; these mixtures could include natural organic matter isolated from surface water and SMP produced by a nitrifying biofilm.

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Abbreviations

2-MIB	2-methylisoborneol
AOA	Ammonia-oxidizing Archaea
AOB	Ammonia-oxidizing Bacteria
BAP	Biomass associated products
BOD	Biochemical oxygen demand
BSA	Bovine Serum Albumin
CCL	Contaminant Candidate List
CDC	Center for Disease Control
CFU	Colony-forming units
COD	Chemical oxygen demand
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EDC	Endocrine-disrupting compounds
EPS	Extracellular polymeric substances
GAC	Granular activated carbon
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
LB	Lysogeny broth
MCL	Maximum contaminant level
N. europaea	*Nitrosomonas europaea*
NOB	Nitrite-oxidizing Bacteria
P. aeruginosa	*Pseudomonas aeruginosa*
PAC	Powdered activated carbon
PPCP	Pharmaceuticals and personal care products
RPM	Revolutions per minute
SDWA	Safe Drinking Water Act
SMP	Soluble microbial products
SPME	Solid-phase microextraction
SRT	Solids retention time
SUVA	Specific ultraviolet absorbance
TrOC	Trace organic contaminants
UAP	Utilization associated products
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
UV	Ultraviolet
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

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CHAPTER 1: INTRODUCTION

1.1 Background

Trace organic contaminants (TrOC) are bioactive and/or persistent chemicals originating from diverse sources that are frequently detected in drinking water sources in the ng/L to µg/L range (Benner et al., 2013). They include compounds such as pharmaceuticals and personal care products (PPCPs), endocrine-disrupting compounds (EDC), plasticizers, flame retardants, pesticides, herbicides, and taste and odor compounds. TrOC regularly enter water bodies by either direct discharge from a point source or runoff during a rain event. Point sources containing TrOC include septic tanks, wastewater treatment plants (WWTPs), and manufacturing effluents. TrOC from diffuse sources (e.g., pesticides and herbicides) become mobile during a rain event, percolating into the ground or running off into a surface water.

Water bodies contaminated with TrOC might also contain ammonia from agricultural runoff (Vitousek et al., 1997; Zhao, Chen, & Zhang, 2008), septic tanks, wastewater treatment plants, dairy lagoons (Shomar, Osenbrück, & Yahya, 2008), or from natural sources. Despite multiple avenues into drinking water sources, ammonia is unregulated in drinking water. Ammonia in drinking water can lead to nitrification, which transforms unregulated ammonia to regulated nitrite and nitrate. Nitrification in the distribution system promotes loss of disinfectant residual (Cunliffe, 1991; Maestre, Wahman, & Speitel, 2013), increased biological growth (Odell et al., 1996; Wilczak et al., 1996; Lipponen, Suutari, & Martikainen, 2002), pathogen regrowth (Wang et al., 2012), taste and odor problems (Suffet et al., 1996), and copper/lead corrosion (Zhang et al., 2009).

Nitrifying biofilters can be used to control ammonia at the treatment plant, which avoids uncontrolled nitrification in the distribution system and degraded drinking water quality; this has been studied at the bench-scale (Laurent et al., 2003; Yapsaklia, Mertoglub, & Çeçenc, 2010) and pilot-scale and full-scale (Lytle et al., 2007). An

increase in TrOC removal at the onset of nitrification has been documented in bench-scale wastewater nitrifying biofilters (Rattier et al., 2014). One potential reason for this improved removal might be related to soluble microbial products (SMP) produced by autotrophic nitrifying bacteria. SMP are organic compounds that are generated during substrate metabolism and biomass decay (Noguera, Araki, & Rittmann, 1994; De Silva & Rittmann, 2000). Some SMP are biodegradable; they can act as the electron donor and carbon source to support heterotrophic bacteria (Kindaichi, Ito, & Okabe, 2004; Merkey et al., 2009). The bacteria that metabolize complex SMP might be primed to degrade other complex carbons, such as TrOC, because of the enzymes they express for SMP degradation.

1.2 Objectives and Approach

The primary objective of this study was to determine if heterotrophic microorganisms acclimated to the SMP produced by nitrifying microorganisms transform TrOC at an increased rate as compared to heterotrophic microorganisms acclimated to a simple organic compound. Here, a mixed heterotrophic culture and a pure culture, *Pseudomonas aeruginosa*, were fed either a simple organic compound (acetate) or SMP. After the cultures were acclimated to these carbon sources, they were spiked with two TrOC, 2-methylisoborneol (2-MIB) and geosmin. The removals of 2-MIB and geosmin in the cultures were examined over time. Thus, this research provides insight into the mechanisms of TrOC removal in drinking-water biofilters.

1.3 Thesis Overview

This thesis focuses on the effect of nitrifier SMP on the removal of TrOC by heterotrophic microorganisms. Chapter 2 reviews the occurrence of TrOC in drinking water, TrOC removal by conventional and biological treatment, and provides an overview of SMP and their impact of TrOC removal. Chapter 3 outlines the materials and methods. Chapter 4 discusses the results of the study. Chapter 5 summarizes the research and outlines areas for future work.

CHAPTER 2: LITERATURE REVIEW

This chapter reviews the literature on the occurrence of trace organic contaminants (TrOC) in raw drinking water and in conventionally and biologically treated drinking water. Subsequently, nitrifying organisms and soluble microbial products (SMP) are reviewed. Finally, the potential impact of SMP on TrOC biodegradation is discussed.

2.1 Trace Organic Contaminants

TrOC, micropollutants, or contaminants of emerging concern are any synthetic or naturally occurring chemical that is not routinely monitored in the environment but are commonly present in waters at trace concentrations (Luo et al., 2014). These compounds are found in water bodies in the low- to sub- parts per trillion. TrOC can be pharmaceuticals, personal care products, pesticides, plasticizers, flame retardants, and surfactants (Richardson & Ternes, 2011). By nature, they are incredibly diverse, as they vary in chemical class, use, intended effect, and source.

Urbanization, increased population, industrialized agriculture, manufacturing emissions, spills, and disposal of waste (e.g., wastewater, landfills) allow TrOC to gain entry to various parts of the environment, including air, soil, oceans, flora, fauna, and drinking water sources. With less than one percent of the earth's water available for human consumption (USGS, 2015), these micropollutants are a public concern. Human and animal wastewater are some of the most important pathways through which TrOC enter the water cycle, making the sources substantial in number, continuously released, and, at times, uncontrolled (Daughton, 2004; Schwarzenbach et al., 2006; Focazio et al., 2008). Every year, industries and manufacturers release approximately 300 million tons of synthetic compounds. Agriculture releases another 140 million tons of fertilizers and pesticides annually (Schwarzenbach et al., 2006). Once these chemicals enter the water cycle, they move into water bodies utilized as drinking water sources. The potential adverse effects on aquatic ecology, humans, and other animals are a growing concern.

2.1.1 Occurrence in Drinking Water

TrOC enter drinking water via two pathways: direct and indirect (Benner et al., 2013). In the direct pathway, TrOC enter a waterway via a point source (e.g., wastewater treatment plant effluent) and are generally of anthropogenic origin. Such TrOC might be down-the-drain personal care products, flushed pharmaceuticals, or chemicals disposed from industrial, commercial, and residential establishments. The wastewater treatment plants receiving these TrOC might not achieve substantial removals, given the diverse properties and low concentrations of the TrOC (Luo et al., 2014). Therefore, TrOC subsequently enter water bodies at these same low concentrations (Metcalfe et al., 2003; Anderson et al., 2004). In the indirect pathway, TrOC enter a waterway via nonpoint source pollution. Nonpoint source TrOC generally originate from chemicals applied to large areas of land (e.g., pesticides, fertilizers, herbicides) that percolate into aquifers or enter a surface water through runoff during a storm event. Additionally, in concentrated animal feeding operations, veterinary medicines are found in the manure that either directly reaches water bodies or enters via runoff from a storm event.

TrOC are ubiquitous in environmental waters, wastewater treatment plant (WWTP) effluents, and in treated drinking water, as reviewed by Benner et al. (2013). Generally, the concentrations of TrOC in raw water, treated water (exiting the treatment plant), and tap water (water exiting the distribution system) are in the low ng/L range. Benotti et al. (2008) sampled raw, treated, and tap water from 19 U.S. drinking water utilities (serving over 28 million people) for a diverse set of pharmaceuticals and endocrine-disrupting compounds (EDC). One target compound was found in each raw water source, and the median concentration was less than 10 ng/L for each TrOC detected. The concentrations of TrOC were lower in treated and tap waters, but it has also been shown that several treated waters have maximum concentrations exceeding 1 μg/L of atrazine, metolachlor, ethanesulfonic acid, lincomycin, sucralose, or nonylphenol (Benner et al., 2013). Furthermore, it is common for there to be a suite of TrOC in a raw water sample. Of 139 streams sampled throughout all regions of the U.S. in 2000, the

median number of TrOC detected in a given water sample was seven out of 95 measured (Kolpin et al., 2002).

2.1.2 Potential Health Effects

TrOC in a drinking water source is a public concern because of their potential health effects. Ingestion of water containing TrOC is a direct route of exposure. However, the term, "trace organic contaminants" is broad, spanning many chemical classes and chemical properties. Their chemical diversity means that there could be a variety of potential health effects. The environmental concentrations found in raw water or treated drinking water are orders of magnitude lower than what would cause an acute negative health effect (Webb et al., 2003; Cunningham, Binks, & Olson, 2009). However, it is unknown if ingesting a suite of chemicals at trace concentrations over a long period, such as would be the case for TrOC ingestion from drinking water, is hazardous. For instance, the majority of studies done on the health effects of pharmaceuticals are based on the effect of a high dose over a short time, in contrast to low-dose chronic ingestion (Snyder et al., 2003; Jones et al., 2005). Further, a drinking water maximum contaminant level (MCL) is based on the toxicity of a specific compound rather than a group of compounds (Stackelberg et al., 2004) that might have synergistic effects.

Several TrOC are classified as EDC, which interfere with development, reproduction, neurological function, and the immune system in animals (NIEHS, 2015). EDC include an expansive list of compounds, including flame retardants, pesticides, and plasticizers (NRDC, 1998). The EU-Strategy for Endocrine Disruptors list of EDC to which humans are likely to be exposed includes 564 compounds, 147 of which are likely persistent in the environment.

2.1.3 Regulations

The Safe Drinking Water Act (SDWA) addresses more than 90 chemicals, including various pesticides, herbicides, and industrial chemicals regulated at trace concentrations. The EPA's Contaminant Candidate List (CCL) also addresses

contaminants that are present at trace concentrations; the CCL-3 is the third such list of contaminants, where the listed compounds are present or anticipated to occur in public water systems and might require future regulation under the SDWA (Richardson & Ternes, 2011). When moving from the CCL to the list of regulated SDWA contaminants, the available health information demonstrates that the removal of the contaminant of interest offers a meaningful opportunity to reduce health risk within the general population as well as those who are at greater risk (i.e., infants, elderly, pregnant women) (Bain, 2014). The CCL-3 contains 104 chemicals and 12 microbiological contaminants: pesticides, disinfection byproducts, waterborne pathogens, pharmaceuticals, and biological toxins. The CCL-4 has been drafted and contains four fewer chemicals than does the CCL-3 (EPA, 2015).

2.1.4 Removal of TrOC by Conventional Drinking Water Treatment

Conventional drinking water treatment typically consists of coagulation, flocculation, sedimentation, filtration, and disinfection. These processes are designed to decrease turbidity and organic carbon and to remove pathogens and common contaminants. The initial intent of these treatment trains was to remove macropollutants from a drinking water source, not to remove TrOC. As discussed below, various studies have investigated how well conventional treatment processes remove TrOC.

In 2004 and 2007, members of the United States Geological Survey (USGS), the Center for Disease Control (CDC), and the New Jersey Department of Environmental Protection monitored the removal of a suite of TrOC through a conventional treatment plant (Stackelberg et al., 2004; 2007). The treatment train of the drinking water treatment plant of interest consisted of the following consecutive processes: clarification, disinfection, and sand/granular activated carbon (GAC) filtration. The chemical properties of the compounds governed which phase of treatment would remove the TrOC. Clarification removed only 15% of the average TrOC concentration from the raw water, disinfection transformed 32% of the average TrOC concentration from the clarified water (by oxidizing the parent compounds) and GAC filtration removed 53% of

the average TrOC concentration (generally the hydrophilic compounds) from the clarified and disinfected water. Both studies detected several TrOC in the finished water that resisted removal with conventional water treatment. Westerhoff et al. (2005) offers that the addition of powdered activated carbon (PAC) and ozone could improve TrOC removal. These additions to a water treatment plant, however, would be costly if the processes were not previously constructed.

2.1.5 Removal of TrOC by Biological Treatment

TrOC biodegradation is a topic of great research interest in drinking water and wastewater treatment. As TrOC encompass a diverse set of compounds, their biodegradability varies (Joss et al., 2006). The rate of degradation also is impacted by the concentration of the contaminant, the solids retention time (SRT), and the class of microorganisms (Clara et al., 2005). TrOC removal has been documented in biological wastewater treatment processes, especially those that incorporate nitrification. For instance, Suarez et al., (2010) measured the degradation rate of 16 pharmaceuticals and personal care products under nitrifying (aerobic) and denitrifying (anoxic) conditions in activated sludge. The compounds were then classified as very highly, highly, moderately, and hardly biodegradable. They noted that the enrichment of activated sludge with nitrifying bacteria removed various compounds that were only moderately biodegradable in conventional activated sludge plants. The degradation kinetics also were faster in the nitrifying system for the majority of the personal care products. Dorival-García et al. (2013) came to a similar conclusion while studying TrOC removal in activated sludge. The addition of nitrifiers to aerobic activated sludge with 6 quinolone antibiotics yielded almost double the removal than in the absence of the nitrifiers (36.2 – 60.0% removal and 14.9 - 43.8% removal of the 6 quinolones, respectively). The role of nitrifiers also was examined in wastewater biofiltration (Rattier et al., 2014). The researchers inhibited nitrification with the compound, allylthiourea, demonstrating that several TrOC were no longer degraded upon inhibition; they concluded that co-metabolism was the main

removal mechanism. These studies all focus on the effect of nitrification on TrOC removal from wastewater.

Drinking-water biofiltration has been shown to be a viable method of removing some TrOC. Over a one-year study period, Zearley & Summers (2012) ran laboratory-scale biofilters fed dechlorinated tap water supplemented with organic matter and 34 commonly occurring TrOC. Of the 34 TrOC, 12 were classified as having a fast or very fast biodegradation rate. The effect of nitrifying organisms on TrOC removal in drinking-water biofilters has yet to be examined.

2.1.6 Representative TrOC, 2-MIB and Geosmin

The two TrOC used in this study were 2-methylisoborneol (2-MIB) and geosmin. Both are naturally occurring taste and odor compounds (Srinivasan & Sorial, 2011) that are produced by some cyanobacteria that bloom in warm temperatures. These compounds are not effectively removed in conventional drinking water treatment processes including coagulation, sedimentation, and abiotic filtration (Srinivasan & Sorial, 2011). However, it has been demonstrated that biofilters are capable of removing these contaminants (Egashira et al., 1992; Ho et al., 2007). Therefore these two TrOC were chosen for this study based on their frequent occurrence in drinking water sources and ability to be biodegraded.

2.2 Nitrifying bacteria

Nitrifying organisms oxidize ammonia (NH_3) to nitrate (NO_3^-). Generally, they are chemolithoautrophic and consume either inorganic ammonia or nitrite to obtain energy to grow (Ward et al., 2011). Nitrification by bacteria is completed in two steps that generally, though not always, are performed by separate sets of microorganisms: ammonia-oxidizing Bacteria (AOB) or ammonia-oxidizing Archaea (AOA) and nitrite-oxidizing Bacteria (NOB). Common AOB genera include *Nitrosomonas, Nitrosococcus,* and *Nitrosospira* (Ward et al., 2011). In 2005, the first AOA was isolated, *Nitrosopumilis*

maritimus (Könneke et al., 2005); over the last 10 years, several other strains have been isolated from marine and soil environments (Stahl & de la Torre, 2012). Common NOB genera include *Nitrobacter*, *Nitrospina*, *Nitrococcus*, *Nitrotoga*, and *Nitrospira* (Ward et al., 2011). First, the AOB or AOA convert ammonia to nitrite,

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad \textbf{Equation 2.1}$$

As shown in Equation 2.1, this requires aerobic conditions. Second, the NOB transform nitrite to nitrate,

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \qquad \textbf{Equation 2.2}$$

Equation 2.2 also demonstrates the requirement for oxygen. The oxidation of ammonia produces protons, thereby causing the pH to drop during nitrification.

2.2.1 Model nitrifier, Nitrosomonas europaea

Nitrosomonas europaea is the most extensively studied and most frequently isolated AOB (Prosser, 1989). The bacterium is relatively fast growing (7- to 8-h doubling time), can withstand high concentrations of ammonium and nitrite, and can be grown in batch cultures and chemostats and on agar plates (Ward et al., 2011). It is a rod-shaped, chemolithoautotrophic bacterium; it uses ammonia for energy production and inorganic carbon for growth. The optimum pH and temperature ranges are 6.0 to 9.0 and 20 to 30°C, respectively (Engel & Alexander, 1958).

2.3 SMP

2.3.1 Definition

SMP are defined as organic compounds that are produced during substrate metabolism and biomass decay (Noguera et al., 1994; De Silva & Rittmann, 2000). Thus, SMP contain electrons and carbon (Laspidou & Rittmann, 2002). The majority of SMP are comprised of humic substances, proteins, and carbohydrates. Their size varies greatly

from <1 kDa to >100 kDa (Jang et al., 2007; Shen et al., 2012). Both heterotrophic and autotrophic organisms can produce SMP (Ni, Rittmann, & Yu, 2011).

Laspidou and Rittmann (2002) published a "unified theory" of SMP that defines SMP as the soluble portion of extracellular polymeric substances (EPS). Aquino and Stuckey (2008) later stated that the unified theory of SMP should also incorporate intracellular components released during cellular lysis or compounds excreted by a cell for some purpose (e.g., compounds in quorum sensing communication). Given their production throughout all stages of a cell's life, SMP generally forms the majority of the effluent chemical oxygen demand (COD) and biochemical oxygen demand (BOD) during biological treatment (Jang et al., 2007), thus making the presence and characteristics of SMP of great interest in biological treatment processes.

2.3.2 Classification

SMP are classified in two groups, based on the bacterial activity from which they originate (Namkung & Rittmann, 1986). The first class of SMP is generated while cells utilize substrate to produce energy and create new cells. This form of SMP is classified as utilization-associated products (UAP). The rate of UAP production is proportional to the rate of substrate utilization and biomass growth; they are small carbonaceous compounds derived from the original substrate (Urbain et al., 1998). The second class of SMP, biomass-associated products (BAP), is generated while active cells undergo endogenous decay, cell lysis, or excrete extracellular compounds. During endogenous decay, the bound EPS and lysed cells are hydrolyzed, thereby creating SMP. Hydrolyzed EPS was initially considered to be the only form of BAP (Laspidou & Rittmann, 2002) until Aquino and Stuckey (2008) proposed that components released from lysed cells and compounds excreted by a cell are SMP as well. The molecular weight of BAP is typically larger than that of UAP, and there is frequently more BAP than UAP (Namkung & Rittmann, 1986; Ni et al., 2010). The sum of the UAP and BAP is the total SMP (Laspidou & Rittmann, 2002; Aquino & Stuckey, 2008). Overall, SMP are a mixture of

undefined organic compounds that cannot be differentiated by simple chemical analysis (Ni et al., 2011).

2.3.3 Biodegradability

SMP can be biodegraded, and the UAP and BAP have different biodegradation kinetics (Rittmann & McCarty, 2001). UAP is more readily biodegraded (De Silva & Rittmann, 2000; Ni et al., 2008). As aforementioned, UAP are smaller compounds that are simpler in structure, making the UAP easier to metabolize. The maximum specific substrate utilization rate for UAP is 1.3 g COD /g volatile suspended solids (VSS)-day, as opposed to 0.07 g COD/g VSS-day for BAP (Laspidou & Rittmann, 2002). These values can occasionally vary, where sometimes BAP can be more degradable than are UAP. Barker et al. (2000) found that under aerobic conditions high-molecular weight SMP (molecular weight >300 kDa, typically BAP) are more biodegradable than are low molecular weight SMP (UAP). Differences in the biodegradability of UAP and BAP among various studies might be due to different reactor types, carbon source concentrations, substrate sources, microbial diversity, type of growth (planktonic or biofilm), and other water quality factors (e.g., pH, dissolved oxygen (DO)). Such differences were showcased in a study by Barker & Stuckey (2001), who used two types of reactors (a continuously stirred tank reactor and a fill-and-draw reactor) to study the effect of feed concentration, feed composition, and reactor volume. These parameters affected the dominant type of SMP and which group of SMP was more biodegradable. Thus, the production of SMP is complex and a function of the environment in which it is produced.

2.3.4 SMP Production by Autotrophs and Heterotrophs

Both autotrophs and heterotrophs produce SMP (Ni et al., 2010). As shown in Table 2.1, heterotrophs produce more SMP than do autotrophs (mass of SMP COD produced per mass of substrate COD utilized, mg COD_p/mg COD_s).

Table 2.1. SMP formation by autotrophs and heterotrophs.

Parameter	Value	Units	Source
UAP formation by autotrophs	0.05	mg COD_p/mg COD_s	Merkey et al. (2009)
EPS formation by autotrophs	0.075	mg COD_p/mg COD_s	Merkey et al. (2009)
UAP formation by heterotrophs	0.12	mg COD_p/mg COD_s	Rittmann & McCarty (2001)
EPS formation by heterotrophs	0.18	mg COD_p/mg COD_s	Laspidou & Rittmann (2002)
Note: Soluble EPS is SMP. EPS is directly proportional to SMP.			

Autotrophs and heterotrophs can live symbiotically; the autotrophs supply the heterotrophs with SMP for use as an electron donor and carbon source (Noguera et al., 1994; Kindaichi, Ito, & Okabe, 2004; Merkey et al., 2009), and the autotrophs receive inorganic carbon from the heterotrophic metabolism of SMP (Ni et al., 2011). Okabe et al. (2005) examined how different phyla of bacteria utilized the products of ^{14}C-labeled nitrifiers (due to their uptake of ^{14}C-labeled bicarbonate). It was shown that members of *Chloroflexi* preferred BAP and *α-Proteobacteria* and *γ-Proteobacteria* preferred UAP. Matsumoto et al. (2010) reaffirmed this by differentiating heterotrophs by which class of autotrophic SMP they consume: UAP, BAP, and BAP that is released during cell lysis (as opposed to the hydrolyzed EPS). On a GAC particle, the UAP was consumed near the surface of the biofilm, as it is generally consumed most readily. The BAP was consumed where the autotrophs cannot access oxygen and lyse or where there exists EPS. Additionally, heterotrophs that utilize nitrifier SMP are most likely K-strategists, which have low maximum specific growth rates (Merkey et al., 2009). Given the relatively slow production of nitrifier SMP, K-strategists (also known as oligotrophs) were shown to be well suited to subsisting on nitrifier SMP because of their ability to survive on low substrate concentrations (Merkey et al., 2009).

2.3.5 Effect of SMP on TrOC Biodegradation

In addition to being a carbon source and electron donor to heterotrophs, SMP might aid in the biodegradation of TrOC in drinking water. Drewes et al. (2014) found that the concentration and composition of the carbon source affected attenuation of TrOC in sandy soil column experiments. They varied the concentrations of easily degradable organic carbon (peptone and yeast extract) and refractory carbons (humic substances). Lower influent biodegradable dissolved organic carbon (DOC) concentrations and greater carbon source complexity resulted in increased microbial community diversity (at the phylum and genus level) which led to greater removal of TrOC. Rauch-Williams et al. (2010) came to a similar conclusion while examining the effect of different bulk organic carbon matrices on TrOC removal in biologically active column experiments. That study underscored the notion that the biodegradation of TrOC requires an oligotrophic metabolism, similar to that of SMP-utilizers.

CHAPTER 3: MATERIALS AND METHODS

3.1 Nitrifying culture

The nitrifying bacterium used for this research was *Nitrosomonas europaea*. This organism was chosen because (1) it has been shown to produce soluble microbial products (SMP) (Kindaichi et al., 2004) and (2) is an autotrophic organism, so it can be grown in the absence of organic carbon. The *N. europaea* Winogradsky ATCC ® 19718™ strain was procured from the American Type Culture Collection (ATCC) on dry ice and subsequently stored at -80°C.

3.1.1 Medium

N. europaea Winogradsky ATCC ® 19718™ was grown in ATCC medium: 2265 (Table 3.1). Each solution was made separately, filtered-sterilized, and stored at room temperature. As needed, the medium components were combined in the appropriate proportions to prepare the overall medium.

Table 3.1. ATCC medium: 2265, *Nitrosomonas europaea* medium.

Solution 1		
Compound	*Unit*	*Value*
$(NH_4)_2SO_4$	g	4.95
KH_2PO_4	g	0.62
$MgSO_4 \bullet 7H_2O$	g	0.27
$CaCl_2 \bullet 2H_2O$	g	0.04
$FeSO_4$ (30 mM in 50 mM EDTA at pH 7.0)	mL	0.5
$CuSO_4 \bullet 5H_2O$	mg	0.2
Distilled water	L	1.2
Solution 2		
KH_2PO_4	g	8.2
NaH_2PO_4	g	0.7
Distilled water	mL	300
Solution 3		
Na_2CO_3 anhydrous	g	0.6
Distilled water	mL	12
Note: Solution 1 (793.6 mL), Solution 2 (198.4 mL), and Solution 3 (7.9 mL) are combined to prepare 1L of ATCC medium: 2265		

3.1.2 Transfers

The *N. europaea* Winogradsky ATCC ® 19718™ culture was initially transferred into an autoclaved test tube containing 10 mL of ATCC medium 2265. The culture was shaken at 150 RPM to provide dissolved oxygen (DO) to the cells. The temperature was maintained at the midpoint of the acceptable range, 25°C. The test tube was wrapped in tin foil to shield the cells from light as they are sensitive to it (Hooper & Terry, 1974). After the development of noticeable turbidity (approximately 14 days), the entire culture was transferred into an autoclaved 250-mL baffled Erlenmeyer flask containing 90 mL of ATCC 2265 medium. Once the culture was growing readily in the Erlenmeyer flask, approximately 10% (by volume) of the culture was transferred to fresh medium every 5 days to ensure substrate availability and an acceptable pH (6-9).

A sterilized biosafety cabinet, Baker SterilGARD® e3, (The Baker Company, Sanford, ME) was used for every transfer. To sterilize the cabinet, it was sprayed with 70% ethanol, wiped down, and all the lab equipment for transfers was placed within it. Next, a 15-minute ultraviolet (UV) light cycle was run to finish sterilizing the cabinet and the equipment.

After several transfers, the culture was stored for up to 4 weeks in the 4°C refrigerator before being transferred into fresh medium (Wahman, 2006). The culture grows more readily after storage in the refrigerator than in a -80°C freezer, allowing for shortened growing times.

3.2 SMP Generation and Characteristics

3.2.1 Bioreactor

To produce nitrifier SMP, a New Brunswick BioFlo 3000 Bioreactor with a 2.5-L working volume (Edison, NJ) was used (Figure 3.1). The bioreactor operated in batch mode and maintained constant pH, temperature, and DO concentration, thereby allowing *N. europaea* to grow to a high concentration.

Figure 3.1. BioFlo 3000 bioreactor.

First, the pH probe within the vessel was removed and calibrated using stock solutions at pH 4.0, 7.0 and 10.0. The vessel was rinsed with distilled water seven times. Next, 1.55 L of freshly prepared Solution 1 (Table 3.1) was added to the vessel and autoclaved. Note that the pH and DO probes were submerged while autoclaved. Once the vessel and medium cooled, 387 mL of filter-sterilized Solution 2 and 15.5 mL of filter-sterilized Solution 3 were added to the vessel, totaling 1.95 L of ATCC medium. The DO probe was calibrated by unplugging the DO cable, setting the DO function to 0, and re-connecting the DO probe.

Next, 50 mL of *N. europaea* culture (either actively growing or recently placed in the refrigerator) was inoculated into the vessel. The vessel was maintained at 26°C, with a DO saturation of 80%, corresponding to a DO concentration of 7 mg/L (USGS, 2015). To maintain the DO concentration, the vessel was agitated at 120 RPM and filtered air (0.2-μm filter) was bubbled into the vessel. The pH was maintained at 7.8 by the

automatic addition of 5% (w/w) filter-sterilized Na_2CO_3 buffer because the pH decreased during nitrification.

3.2.2 SMP Isolation

Since DO and the autotrophic carbon source, carbonate, were continually supplied to the vessel, ammonia was the limiting substrate (initial concentration of 694 g NH_4^+-N/L). Once the culture in the vessel was turbid, a sample was taken through the sampling port on the vessel by suctioning out at least 10 mL of culture. A raw sample was analyzed with an ammonia probe (3.4.2). This was done daily until the concentration of ammonia was below 1 mg NH_4^+-N/L, which took approximately 10 days. At this point, the two liters of culture were removed from the vessel and filtered through a 0.2-μm filter. An aliquot was removed for dissolved organic carbon (DOC) analysis (3.4.3). Aliquots also were removed to measure chemical oxygen demand (COD) (3.4.11), the major cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (3.4.6), specific ultraviolet absorbance (SUVA) (3.4.13), and alkalinity (3.4.7). The rest of the filtrate (containing SMP) was stored in 50-mL sterile Falcon tubes in the -20 °C freezer. To ensure that the organic matrix was similar before and after freezing, UV absorbance at 254 nm was measured in triplicate before and after storage.

3.2.3 SMP Biodegradability

The biodegradability of the SMP produced was assessed by growing a culture of *Pseudomonas aeruginosa* (3.3.3) on SMP as the sole carbon source and electron donor as compared to using acetate as the sole carbon source and electron donor. Two flasks containing 300 mL of synthetic water (3.3.2) were supplemented with 1.5 mg/L soluble C of either SMP or acetate and inoculated with a dense culture of *P. aeruginosa*. At three time points (0, 24, 48 hours), unfiltered samples were used to measure the total organic carbon (TOC) and viable plate counts (3.4.3 and 3.4.8, respectively).

3.3 Trace Organic Contaminant Degradation Experiments

3.3.1 Overview

These experiments were conducted to examine the rate of transformation of two TrOC, 2-methylisoborneol (2-MIB) and geosmin. This was done by acclimating a pure culture, *P. aeruginosa* PAO1, and a mixed heterotrophic community, to two carbon sources: SMP and acetate. Additionally, there were two controls: cells grown on SMP or acetate and inhibited by sodium azide addition, and a no-biomass control (Table 3.2). The carbon source and electron donor supplied to each culture was 20 mg/L soluble COD.

Table 3.2. Experimental matrix for assessing the effect of nitrifier SMP on TrOC removal.

Culture #	Acclimation to Carbon Source	Inoculum	Notes
1	SMP	*P. aeruginosa*	
2	Acetate	*P. aeruginosa*	
3	SMP	*P. aeruginosa*	Killed control (sodium azide addition after acclimation (3.3.3.1))
4	SMP	Mixed heterotrophs	
5	Acetate	Mixed heterotrophs	
6	Acetate	Mixed heterotrophs	Killed control (sodium azide addition after acclimation (3.3.4.1))
7	None	None	No-biomass control (synthetic water medium was used)

The experiment consisted of two phases: carbon source acclimation and TrOC biodegradation. First, all the cultures were given 3-7 days to acclimate to metabolizing the supplied carbon source (3.3.3.1 and 3.3.4.1). Next, each culture was spiked with 2-

MIB and geosmin (as the sole carbon and electron donor) and sampled at various time-points over the next 4-7 days to assess loss of the compounds (3.3.7).

3.3.2 Synthetic Drinking Water

The water used for the carbon source acclimation phase and the TrOC biodegradation phase was synthetic water with the following composition (Table 3.3).

Table 3.3. Synthetic water medium.

Compound	**Final concentration in the medium (mg/L)**
Carbonate	
$NaHCO_3$	84.01
Salts	
Na_2SO_4	17.75
NaCl	13.68
$CaCl_2 \cdot 2H_20$	2.81
$MgCl_2 \cdot 6H_20$	3.88
Trace Metals	
$AlCl_3 \cdot 6H_2O$	0.2
$CoCl_2 \cdot 6H_2O$	0.0382
$CuSO_4 \cdot 5H_20$	0.0574
H_3BO_3	0.0303
$MnCl_2 \cdot 4H_2O$	0.2807
$Na_2MoO_4 \cdot 2H_2O$	0.0254
Na_2SO_4	0.142
$NiCl_2 \cdot 6H_2O$	0.0216
$ZnSO_4 \cdot 7H_2O$	0.288
$FeSO_4 \cdot 7H_2O$	0.9099[1]
Vitamins	
B12	0.001
Biotin	0.02
Calcium pantothenate	0.05
Folic acid	0.02
Nicotinamide	0.05
P-aminobenzoic acid	0.05
Pyridoxine-HCl	0.1
Riboflavin	0.05
Thiamine-HCl	0.05

[1]: This corresponds to 59.6 µL of 30 mM $FeSO_4$ and 50 mM EDTA (at pH 7.0) solution per liter of medium.

The synthetic water consisted of a carbonate buffer, amended with salts (based on the composition of a groundwater in Rialto, CA) (Li et al., 2010), vitamins (Staley, 1968), and trace metals (London et al., 2011). The carbonate and salt solutions were prepared at 100x concentrations, and the trace metals and vitamin solutions were prepared at 1000x concentrations; each of these solutions was made separately and then filter-sterilized. When preparing the synthetic water, Nanopure water (3.4.1) was autoclaved, cooled and amended with each solution to the final concentrations shown in Table 3.3.

A carbon source and electron donor was added to the synthetic water medium to a final concentration of 1.5 mg/L soluble C for the biodegradation experiment (3.2.3) and 20 mg/L soluble COD for the trace organic contaminant degradation experiment (3.3). The carbon source and electron donor was either acetate (simple carbon) or SMP.

3.3.3 P. aeruginosa Inoculum

P. aeruginosa is a gram-negative, rod-shaped bacterium that is found ubiquitously in the environment – including drinking water sources (Stover et al., 2000). *P. aeruginosa* is capable of transporting, metabolizing and growing on a diverse array of organic substances, which makes it amenable to surviving in a variety of environments (Stover et al., 2000). Given the complex and diverse metabolic capabilities of *P. aeruginosa*, it is a model organism for use in this study.

The *P. aeruginosa* strain used was PAO1. An aliquot was taken from a freezer stock and grown on a Lysogeny broth (LB) plate for approximately 18 hours at 37 °C. A single colony was taken and inoculated to 10 mL of LB medium and grown aerobically with shaking (200 RPM) at 37 °C. Once the culture was visibly turbid, a 7-mL aliquot was retrieved and centrifuged for 5 minutes at 5,000 x *g*. The supernatant was decanted, and the pelleted cells were resuspended in 7 mL of a buffer (8 mM K_2HPO_4 + 10 mM Na_2CO_3) and centrifuged again. Then, the cells were resuspended in 7 mL of synthetic water (3.3.2) and used as the inoculum for the TrOC degradation study. Cell concentration was examined by viable plate counts (3.4.8) and protein concentration (3.4.10).

3.3.3.1 Carbon Acclimation

Three 250-mL Erlenmeyer flasks were prepared containing 150 mL of sterile synthetic water medium (Table 3.3). Cultures 1-3 were supplied 20 mg/L of carbon source (COD). Cultures 1 and 3 were supplied SMP as the carbon source, and Culture 2 was supplied acetate as the carbon source (Table 3.2). The flasks were topped with Morton closures and placed in a shaking incubator (200 RPM) at 25°C. Every 2 days, the culture was filtered through a 0.2-µm filter, washed with a buffer (8 mM K_2HPO_4 + 10 mM Na_2CO_3), and each filter with its retained biomass was placed into a new flask with fresh synthetic medium containing the carbon source at 20 mg/L soluble COD. After incubating for at least 2 hours (200 RPM) at 25°C, the filters were aseptically removed from the flasks.

3.3.3.2 Kinetic Study Preparation

Four 150-mL serum bottles were supplied with 130 mL of carbon-free fresh synthetic water medium (3.3.2). The conductivity was normalized between the four bottles due to the varying conductivities during the carbon acclimation phase with SMP or acetate (3.3.3.1). The cells acclimated to SMP were used to a high conductivity since the SMP were produced in a high conductivity solution (4.2.1). To normalize the conductivity between the cultures grown on acetate and SMP (low conductivity and high conductivity, respectively), each bottle was supplemented with 336 mg/L Na_2CO_3 and 50 mg/L K_2HPO_4 to produce a mid-range conductivity. The *P. aeruginosa* cells, acclimated to SMP or acetate, were spun down at 5,000 x *g* for 5 minutes. The supernatant was decanted so only the cells remained. The cells were resuspended in the synthetic water. A sample was taken from Culture 1 - 3 to measure viable plate counts and protein concentration (3.4.8, 3.4.10). Based on protein concentration, the biomass contents were normalized across all bottles; then, sodium azide was added to Culture 3. Each bottle was spiked to a final concentration of 930 ng/L of 2-MIB and 400 ng/L of geosmin (Sigma-Aldrich, St. Louis, MO) and topped with a butyl rubber stopper. The bottles were shaken vigorously to distribute the 2-MIB and geosmin evenly throughout the medium.

3.3.4 Mixed Heterotrophic Inoculum

An activated sludge (AS) sample was retrieved from the Walnut Creek Wastewater Plant (Austin, TX). Triplicate aliquots of sludge were processed to prepare a heterotrophic inoculum for each heterotrophic degradation experiment. First, 7 mL of sludge was filtered through a 20-µm filter (Whatman Grade 41 Ashless Filters, GE Healthcare, United Kingdom) to remove very large particles. The filtrate was then filtered through a 0.2-µm filter and washed with a buffer (8 mM K_2HPO_4 + 10 mM Na_2CO_3). The washed cells retained on the 0.2-µm filter were used as the mixed culture inoculum.

3.3.4.1 Carbon Acclimation

Three 1000-mL Erlenmeyer flasks were prepared containing 350 mL of sterile synthetic water medium (Table 3.3). Culture 4 was supplied SMP as a carbon source, and Cultures 5 and 6 were supplied acetate (Table 3.2). Similar to the *P. aeruginosa* acclimation, the carbon source was added to a final concentration of 20 mg/L soluble COD. A filter containing washed heterotrophic cells (3.3.4) was placed into each flask, topped with sterile foam plugs and shaken in an incubator for at least 2 hours (200 RPM) at 25°C before the filters were aseptically removed. To supply fresh carbon, every 2 days the culture was filtered through a 0.2-µm filter, washed with a buffer (8 mM K_2HPO_4 + 10 mM Na_2CO_3), and each filter with its retained biomass was placed into a new flask with fresh synthetic medium containing the carbon source at 20 mg/L soluble COD. After incubating for at least 2 hours (200 RPM) at 25°C, the filters were aseptically removed from the flasks.

3.3.4.2 Kinetic Study Preparation

Four 150-mL serum bottles were supplied with 130 mL of carbon-free fresh synthetic water medium (3.3.2). The conductivity was normalized between the four bottles due to the varying conductivities during the carbon acclimation phase (3.3.4.1). The cells acclimated to SMP were used to a high conductivity since the SMP were produced in a high conductivity solution (4.2.1). To normalize the conductivity between the cultures grown on acetate and SMP (low conductivity and high conductivity,

respectively), each bottle was supplemented with 336 mg/L Na_2CO_3 and 50 mg/L K_2HPO_4 to produce a mid-range conductivity. Next, the acclimated heterotrophic cells were filtered through a 0.2-μm filter, washed with carbon-free synthetic water, and placed into a serum bottle with 130 mL of fresh carbon-free synthetic water. The bottles were shaken vigorously until all visible biomass was dislodged from the filters, which were then removed aseptically. A sample was taken from Cultures 4 - 6 to measure heterotrophic plate counts and protein concentration (3.4.8, 3.4.10). Based on protein concentration, the biomass was normalized across all bottles; then, sodium azide was added to Culture 6. Heterotrophic plate counts also were measured for the duration of the experiment. Each bottle was spiked to a final concentration of 530 ng/L of 2-MIB and 280 ng/L geosmin (Sigma-Aldrich, St. Louis, MO) and topped with a butyl rubber stopper. The bottles were shaken vigorously to distribute the 2-MIB and geosmin evenly throughout the medium.

3.3.6 Controls

The controls in this study were Cultures 3, 6, 7 (Table 3.2). During the acclimation period (3.3.3.1 and 3.3.4.1), Culture 3 was identical to Culture 1, and Culture 6 was identical to Culture 5. After the acclimation period, 1 g/L of sodium azide was added to Cultures 3 and 6. Azide inhibits the respiratory chain by stopping oxidative phosphorylation, therefore impeding metabolism (Ishikawa, Bao-Li, & Hitoshi, 2006). This was to differentiate if the losses of 2-MIB and geosmin were due to biodegradation or other mechanisms (e.g., sorption to cells). Culture 7 was a no-biomass control, which was used to determine if 2-MIB and geosmin losses were due to sorption to the glassware, volatilization, or other mechanisms.

3.3.7 Sampling

Immediately after mixing the 2-MIB and geosmin in the serum bottles, a 10-mL sample was taken for analysis (t_0) and then periodically over the next four days. The bottles were kept in a shaking incubator (150 RPM) at 25°C. The samples were taken with autoclaved acid-washed glass pipettes. When each sample was taken, it was spiked

with 5 µL of 200 µg/L geosmin internal standard (Sigma Aldrich, St. Louis, MO). Ten percent of the samples taken were duplicates. The samples were analyzed on the Gas Chromatograph Mass Spectrometer (GC-MS) within one day of sampling in an effort to minimize losses (3.4.14). If the samples needed to be stored, they were kept at 4ºC.

Five days after spiking with TrOC, 20 mg/L COD SMP was added to Culture 4, and acetate was added to Culture 5. These carbon spikes were to determine if the removal of 2-MIB and geosmin required the presence of a primary substrate (as would be needed for co-metabolism).

3.4 Analyses

3.4.1 Nanopure Water

High-quality water was supplied through the Barnstead™ Nanopure™ system (Thermo Fisher Scientific, Waltham, MA). First, distilled water produced on the University of Texas at Austin campus was fed through a Barnstead B-Pure™ Water Purification System; then it was fed through a DIamond Kit Type 1 R/O & Distilled Feed, which is for Low Organics, Type 1 Water, Reverse Osmosis or Distilled Water applications. The finished water had a resistivity of 18.2 MΩ•cm and a conductivity of 0.055 µS/cm.

3.4.2 Ammonia

Ammonia was measured with an ion selective probe, YSI TruLine Ammonia Electrode, connected to a meter (Xylem, Rye Brook, NY). The ammonia probe was calibrated daily. Four calibration standards (2.0, 1.0, 0.5, and 0.1 mg/L-N) were prepared in volumetric flasks using Nanopure water and a 10 mg/L-N ammonia stock (Hach Company, Loveland, CO). For sample analysis, 200 µL of pH-adjusting Ionic Strength Adjustor solution was added to a 10-mL sample (suggested ratio by manufacturer) to ensure that all ammonium ions (NH_4^+) were converted to ammonia (NH_3), and the measurement was performed immediately in a stirred vessel.

3.4.3 DOC and TOC

DOC/TOC were measured with Shimadzu TOC-L (Shimadzu Corp., Kyoto, Kyoto Prefecture, Japan). Potassium hydrogen phthalate was used to prepare standards for analysis (Sigma-Aldrich, St. Louis, MO). A sample of potassium hydrogen phthalate was dried for one hour at 105 ºC and then used to prepare a 200 mg/L C stock solution. The stock solution was stored in an acid-washed and autoclaved amber glass bottle. The stock solution was diluted to create a 5 mg/L C standard. The TOC-L Analyzer diluted the 5 mg/L C standard to prepare a standard curve (0, 0.5, 0.75, 1, 2, 3, 4, 5 mg/L C). The water used for dilutions was Nanopure water. All samples taken for analysis were placed into acid-washed 40-mL vials.

3.4.4 pH

The pH was measured with an Orion 720A pH ISE mV Oxidation Reduction Potential Meter (Thermo Fisher Scientific, Waltham, MA). The pH meter was calibrated prior to use with a three-point calibration using standards for pH 4, 7, and 10. At least 10 mL of sample was collected for measurement. The pH was measured directly from the vial used for sampling and continuously stirred throughout the measurement. The vial only was opened immediately before analysis.

3.4.5 UV_{254}

UV absorbance at 254 nm was measured with the Agilent 8453 UV-visible Spectroscopy System (Agilent Technologies, Santa Clara, CA). The cuvettes were quartz, and the path length was 1 cm. Prior to measuring the samples, a blank consisting of Nanopure water was read. The cuvette was flushed with sample twice and then read.

3.4.6 ICP-OES

The major cations, Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}, were measured using a Varian 710-ES ICP Optical Emission Spectrometer (Varian, Palo Alto, CA). The instrument was calibrated with a 5-point calibration curve prior to each run. The standards were prepared using 1000 mg/L standard stock solutions (RICCA Chemical Company, Arlington, TX)

and Nanopure water. The working standards for the calibration curve for each cation were 0.1, 0.5, 1, 3 and 5 mg-cation/L.

3.4.7 Alkalinity

Alkalinity was determined according to Standard Methods 2320. A 30-mL sample of SMP (3.2) was titrated with 3 N HCl until the pH reached 4.5. The alkalinity was then calculated as follows:

$$Alkalinity\left(\frac{mg\ CaCO_3}{L}\right) = \frac{A \times N \times 50{,}000\left(\frac{mg\ CaCO_3}{eq}\right)}{V} \qquad \textbf{Equation 3.1}$$

where

A = volume of standard acid used (mL)

N = normality of standard acid (eq/L)

V= sample volume (mL)

3.4.8 P. aeruginosa Viable Plate Counts

Viable plate counts were used to estimate the number of cells in a sample containing *P. aeruginosa*. First, LB plates were prepared by pouring 15 mL of medium into 100 ×15 mm presterilized disposable polystyrene petri dishes. After drying at room temperature for at least twelve hours, the plates were inverted with lids on and stored in plastic bags at 4^{o}C. The counters were wiped with 70% (v/v) ethanol, and plating was conducted near a flame to prevent contamination. A 10x phosphate buffered saline (PBS) solution was prepared (Table 3.4); from it, a 1x PBS solution was prepared and filter-sterilized.

Table 3.4. 10x PBS buffer (500 mL total volume).

Compound	Mass (g)
NaH_2PO_4	0.156
Na_2HPO_4	0.52
NaCl	0.453

Aliquots of 0.9 mL of 1x PBS buffer were placed into several 2-mL tubes. Next, 0.1 mL of sample was added to the first tube of 1x PBS, and serial dilutions were performed (through 10^{-6}). Aliquots (10 μL) were spot-plated in triplicate from each dilution. When the spots were dry, the plates were inverted and wrapped with parafilm.

Spots containing the desired number of colonies (between 10 and 40) were counted after incubation at 37 ºC for 24 hours. Colony Forming Unit (CFU) and the dilution factor of each spot were recorded, and CFU/mL was calculated according to the following equation:

$$CFU/mL = \frac{Colonies\ counted \times Dilution\ factor}{0.01\ mL} \qquad \textbf{Equation 3.2}$$

3.4.9 Heterotrophic Plate Counts (HPC)

HPC was determined according to Standard Methods 9215. R2A plates (HiMedia, Mumbai, India) were prepared by pouring 15 mL of agar medium into 100 ×15 mm presterilized disposable polystyrene petri dishes. Test tubes containing 9 mL of sterile 1x PBS were prepared and labelled for serial dilutions. A 1-mL aliquot of raw sample was added to the first tube of 1x PBS, and serial dilutions were prepared (through 10^{-5}). Aliquots (50 μL) were spread-plated from each dilution. When plates were dry, they were inverted and wrapped with parafilm; they were incubated at room temperature (approximately 23°C) for 7 days.

Plates yielding an appropriate number of colonies (30 to 300 colonies) were chosen for counting. CFU and the dilution factor of each plate were recorded, and CFU/mL was calculated with the following equation:

$$CFU/mL = \frac{Colonies\ counted \times Dilution\ factor}{0.05\ mL} \qquad \textbf{Equation 3.3}$$

3.4.10 Protein Analysis

The concentration of proteins was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). A six-point calibration curve was made using Bovine Serum Albumin (BSA) standards supplied in the kit for a working range of 5–250

µg/mL. To prepare the samples for analysis, a 1-mL sample aliquot was centrifuged at 5,000 x *g* for 5 minutes to pellet the cells. The supernatant was decanted, and the cells were resuspended in a buffer (8 mM K_2HPO_4 + 10 mM Na_2CO_3); the cells were again centrifuged at 5,000 x *g* for 5 minutes, the supernatant decanted, and the cells resuspended in 3 mL of cell lysis buffer (Table 3.5). Finally, the cells were sonicated with a 0.5-inch probe sonicator (Thermo Fisher Scientific, Waltham, MA) for 5 minutes at 15%.

Table 3.5. Cell lysis buffer.

Chemical	Unit	Value
Tris base	g	0.606
NaCl	g	0.584
Glycerol	mL	5
Nanopure water	mL	95
Note: HCl added to adjust pH to 7.5		

3.4.11 COD

COD was measured with CHEMetrics LR COD 0-150 mg/L kit (CHEMetrics Inc, Midland, VA). Samples that were not analyzed immediately were acidified with concentrated sulfuric acid to a pH of < 2 and stored at 4ºC for no longer than 28 days. To measure COD, the samples were first mixed vigorously to homogenize. Two mL of sample was added to a CHEMetrics COD vial, shaken, and placed on a digestor block for 2 hours at 150 ºC. After the vials had cooled, a sample was poured into a 1-cm plastic cuvette, and the UV absorbance at 420 nm was measured with the Agilent 8453 UV-Visible Spectroscopy System (Agilent Technologies, Santa Clara, CA). The absorbance value was converted to mg/L COD by the CHEMetrics calibration equation (Equation 3.4).

$$COD\left(\frac{mg}{L}\right) = -331(abs) - 0.6 \qquad \textbf{Equation 3.4}$$

3.4.12 Conductivity

Conductivity was measured in the Ultrameter III (Myron L Company, Carlsbad, CA). The measurement cell in the instrument was rinsed twice with Nanopure water, and then the conductivity of the sample was measured.

3.4.13 SUVA

The SUVA of the SMP was calculated from the following equation:

$$SUVA = \frac{UV_{254nm}\,(cm^{-1}) \times 100\left(\frac{cm}{m}\right)}{DOC\left(\frac{mg}{L}\right)} \qquad \textbf{Equation 3.5}$$

where,

UV_{254} = absorbance at 254 nm

DOC = dissolved organic carbon concentration

UV_{254} was measured as described in 3.4.5. Samples were filtered (through a polyethersulfone 0.45-µm filter), and DOC was measured as described in 3.4.3.

3.4.14 Gas Chromatograph and Mass Spectrometer (GC-MS)

The TrOC, 2-MIB and geosmin, were measured with the gas chromatograph Agilent 7890B (Agilent Technologies, Santa Clara, CA) and mass spectrometer Agilent 5977A MSD. Prior to creating standards for sampling, amber 15-mL Agilent vials were acid-washed, dried, and filled with 3 g of NaCl. A six-point calibration curve was prepared for both 2-MIB and geosmin at 20, 50, 100, 300, 500, and 1000 ng/L each. These standards were created by serially diluting a 100 µg/mL (each) geosmin and 2-MIB solution in Nanopure water. Samples were taken in 10-mL aliquots and amended with 5 µL of 200 µg/L (±)-Geosmin ≥97% internal standard (Sigma-Aldrich, St. Louis, MO), resulting in 100 ng/L internal standard in each sample. Given that the concentration of the internal standard was known, it allowed for a comparison of the internal standard to the 2-MIB and geosmin reading in each sample to accurately assess how much analyte was present during analysis. The high salinity (300 g/L NaCl) in each sampling vial ensured

there was no additional biological degradation by the microorganisms. Once the samples were taken, they were either immediately run on the GC-MS or kept at 4 ºC for no more than 96 hours.

The samples were analyzed in the program GC-MS Data Analysis. The quantifying ions for 2-MIB and geosmin (95 and 112, respectively) were extracted individually, and the peaks were integrated. The area under the peaks corresponded to the concentration of analyte. This was repeated with the internal standard (quantifying ion of 115), which allowed for each sample to be normalized.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Nitrifying culture

To ensure the *Nitrosomonas europaea* Winogradsky ATCC ® 19718™ strain was oxidizing ammonium and thereby producing hydrogen ions (Equation 2.1), the pH was taken over time for three *N. europaea* batch cultures (Figure 4.1). The pH decreased as expected due to ammonium oxidation (Equation 2.1).

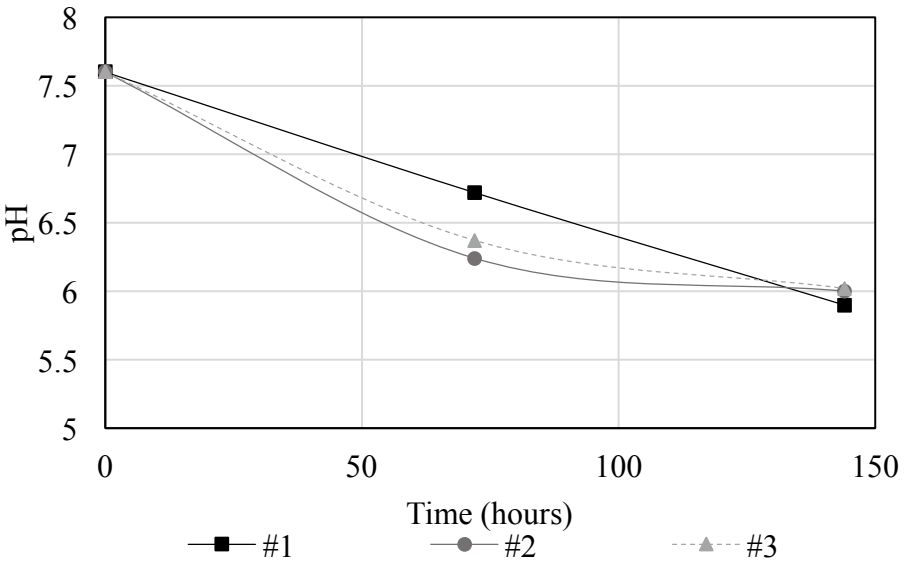

Figure 4.1. pH of *N. europaea* cultures over time.

4.2 Soluble Microbial Products (SMP) Isolation

4.2.1 SMP Characterization

After SMP was produced by *N. europaea*, the SMP solution was characterized for alkalinity, dissolved organic carbon (DOC), specific ultraviolet absorbance (SUVA), chemical oxygen demand (COD), conductivity, and major ion concentrations (Table 4.1).

Table 4.1. SMP characterization.[1]

Parameter	Unit	Value
Alkalinity	mg/L $CaCO_3$	2,360
Conductivity	µS	3,701
DOC	mg/L	7.5
COD	mg/L	100
UV_{254}	cm^{-1}	0.620
SUVA	L/mg-m	0.083
Na^+	mg/L	3,056
K^+	mg/L	1,216
Ca^{2+}	mg/L	6.25
Mg^{2+}	mg/L	7.9
Fe^{3+}	mg/L	0.14
[1]Multiple batches of SMP were produced throughout this study. These values characterize the most recent batch of SMP that was used for every experiment herein.		

As previously mentioned in 3.2.1, Na_2CO_3 was pumped into the bioreactor to raise the pH and supply carbon to *N. europaea* as ammonium was oxidized. According to Equation 2.1, oxidizing 694 mg/L NH_4^+-N (as the bioreactor operated from a concentration of 694 mg/L NH_4^+-N to below 1 mg/L NH_4^+-N), produces 0.099 M of H^+ ions. By using Na_2CO_3 to maintain the pH at 7.8, the SMP solution had high values for Na^+ concentration, alkalinity, and conductivity.

The SUVA value is indicative of the aromaticity of the organic matter in a sample. Generally, high SUVA values, > 4 L/mg-m, are indicative of hydrophobic, aromatic, high molecular weight compounds (i.e., humic acids), whereas low SUVA values, < 2 L/mg-m, indicate the presence of hydrophilic, aliphatic, low molecular weight compounds (Edzwald & Van Benschoten, 1990; White et al., 1997; Weishaar et al., 2003). The SMP SUVA value of 0.083 L/mg-m reflects the latter characteristics. The SUVA of the SMP produced in this study is considerably lower than values found for other SMP in the literature. In a synthetic wastewater batch reactor, the SUVA of SMP

ranged from 0.65 to 0.92 L/mg-m (Jarusutthirak & Amy, 2006). Jarusutthirak and Amy (2006) stated the majority of the SMP in their batch reactor were biomass-associated products (BAP) produced from cell lysis and characterized by a high molecular weight. The SUVA value obtained in this study might be low due to minimal production of BAP from cell lysis. The *N. europaea* cells grown in the bioreactor always had substrate so cell death was most likely minimal.

4.2.2 SMP Storage

Ultraviolet absorbance (UV) at a wavelength of 254 nm was measured for the first batch of SMP before and after storage at -20°C. The UV absorbance before freezing was 0.653 and after freezing/thawing was 0.662. The 1.3% difference in UV before and after storage suggests that a freeze-thaw cycle did not substantially alter the aromaticity of the SMP.

4.2.3 SMP Biodegradation

Pseudomonas aeruginosa was acclimated to SMP or acetate over a 48-h period (Figure 4.2). During this time, the *P. aeruginosa* concentration decreased from its initial value of 4.5×10^8 colony-forming units (CFU)/mL. The loss in biomass can be attributed to the small quantity of carbon source and electron donor supplied to the microorganisms. Prior to this experiment the cells were grown in Lysogeny broth (LB), which is nutrient rich and resulted in a high biomass concentration. Resuspending the cells in a nutrient-poor synthetic water likely caused cell death due to low concentrations of carbon.

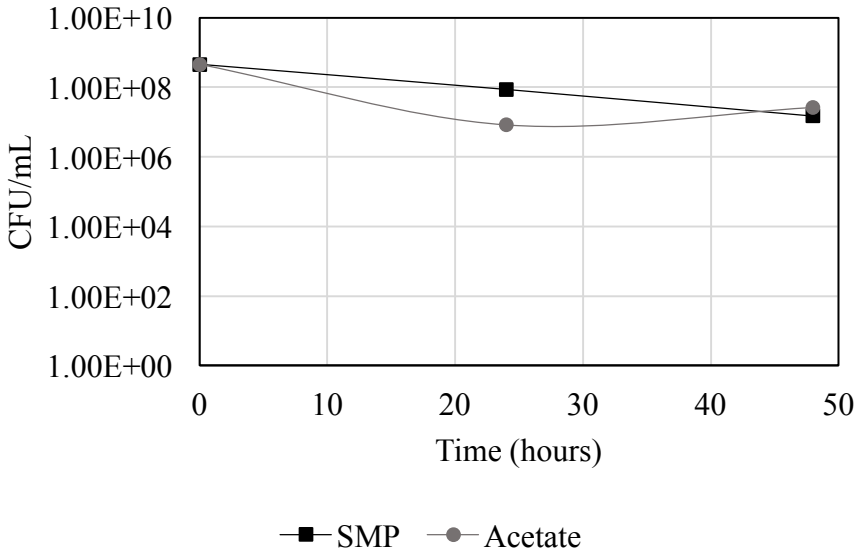

Figure 4.2. *P. aeruginosa* concentration over time during the acclimation to SMP or acetate. Initial conditions: 4.5 x 10^8 CFU/mL, and 1.5 mg/L soluble C from SMP or acetate.

Figure 4.3 shows the TOC in the *P. aeruginosa* cultures over time. Since TOC was measured (no filtration of samples), a portion of the TOC is attributable to biomass. Prior to inoculating, 1.5 mg/L soluble C was supplied to each flask as carbon source and electron donor; therefore, at time 0, 1.21 mg/L and 1.45 mg/L C of biomass was present in the acetate and SMP flasks, respectively. Although the individual contributions of biomass and the SMP/acetate to TOC were not assessed after time 0 (only aggregate TOC was measured), the data suggest that more acetate TOC was consumed by *P. aeruginosa* as compared to the amount of SMP TOC consumed by *P. aeruginosa*. This is because the biomass concentrations between the two cultures were indistinguishable from one another at 48 hours (Figure 4.2), but the residual TOC in the acetate culture was substantially lower than that in the SMP culture at that time (Figure 4.3). This result might be expected given the simplicity of acetate as a carbon source and electron donor. Additionally, taken together, the TOC and biomass data suggest that acetate/SMP consumption largely took place during the first 24 hours. Based on these results, the carbon source (acetate or SMP)

was replaced every 48 hours during the carbon acclimation portion of this study (3.3.3.1 and 3.3.4.1).

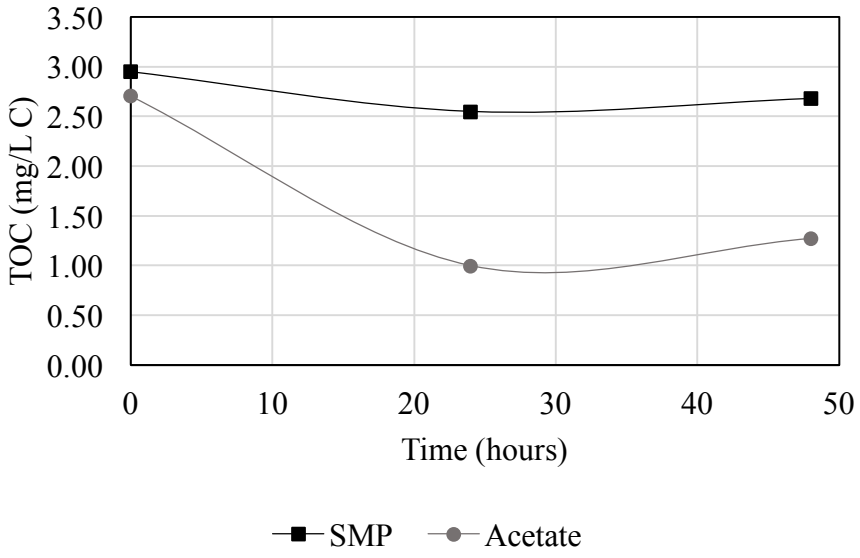

Figure 4.3. TOC in *P. aeruginosa* cultures over time during the acclimation to SMP or acetate. Initial conditions: 4.5 x 10^8 CFU/mL, and 1.5 mg/L soluble C from SMP or acetate.

4.3 Trace Organic Contaminant Kinetic Study

The next experiments were conducted to examine the rate of transformation of two TrOC, 2-methylisoborneol (2-MIB) and geosmin between cultures that were acclimated to acetate (simple compound) or SMP. *P. aeruginosa* PAO1 and a mixed heterotrophic community were acclimated to two carbon sources: SMP or acetate. Additionally, there were two controls: cells grown on SMP or acetate and inhibited by sodium azide addition, and a no-biomass control.

These experiments consisted of two phases: carbon source acclimation and TrOC biodegradation. First, all the cultures were given 3-7 days to acclimate to metabolizing the supplied carbon source (3.3.3.1 and 3.3.4.1). Next, each culture was spiked with 2-

MIB and geosmin and sampled at various time-points over the next 4-7 days to assess loss of the compounds (3.3.7).

4.3.1 Biomass Normalization

After the three flasks containing *P. aeruginosa* and SMP or acetate were acclimated to their respective carbon source, centrifuged, and resuspended in 130 mL of synthetic water without carbon, the protein concentration was measured (Table 4.2) as a proxy for biomass concentration.

Table 4.2. Protein concentration of *P. aeruginosa* cultures acclimated to acetate and SMP.

Culture #	Carbon Source	Protein Concentration Measured in Undiluted Culture (mg BSA/mL)	Calculated Protein Concentration (mg BSA/mL) in Diluted Culture
1	SMP	33.4	-
2	Acetate	43.2	33.4
3	SMP	33.8	-

Given these biomass concentrations, 30 mL of Culture 2 was removed and replaced with the same volume of synthetic water to normalize the biomass among all flasks. After this dilution, the viable plate counts from Culture 1 and Culture 2 were 1.5 x 10^7 and 2.6 x 10^7 CFU/mL, respectively, indicating that protein concentration was an acceptable proxy for biomass; the calculated protein concentration for Culture 2 is shown in Table 4.2.

Similar to the *P. aeruginosa* experiments, activated sludge (AS) was acclimated to SMP or acetate and then resuspended in synthetic water. The protein concentration was 42 mg BSA/mL for all AS cultures. This protein concentration corresponded to heterotrophic plate counts (HPC) of 1.4 x 10^6 for the cells acclimated to SMP and 4.2 x 10^6 for cells acclimated to acetate. To confirm that the number of cells was similar over

the duration of the experiment, HPC were monitored throughout the TrOC degradation study (Figure 4.4).

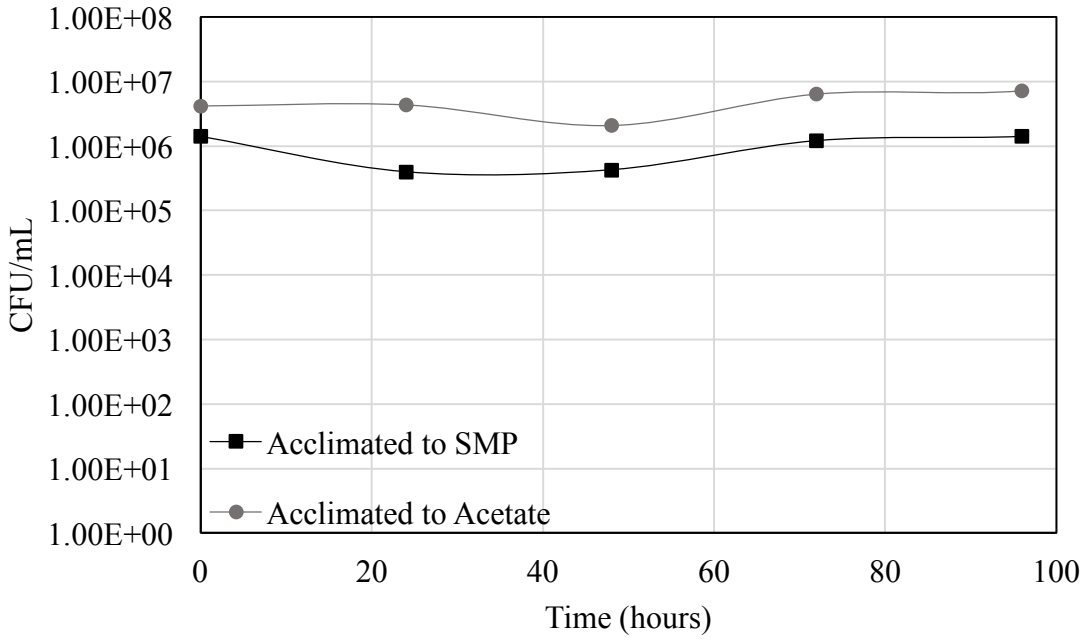

Figure 4.4. HPC in the AS cultures throughout the TrOC study (4.3.3). Initial conditions: 530 ng/L 2-MIB, 280 ng/L geosmin, 1.4 x 10^6 CFU/mL of cells acclimated to SMP (20 mg/L soluble COD), 4.2 x 10^6 CFU/mL of cells acclimated to acetate (20 mg/L soluble COD).

As shown, the concentrations of heterotrophic cells in both flasks were reasonably similar to each other for the length of the study.

4.3.2 Removal of 2-MIB and Geosmin by P. aeruginosa

After acclimation to acetate or SMP, the washed and resuspended *P. aeruginosa* were placed into synthetic water and spiked with 2-MIB and geosmin as the sole carbon and energy sources. The concentration of 2-MIB in the bottles fluctuated over time (Figure 4.5).

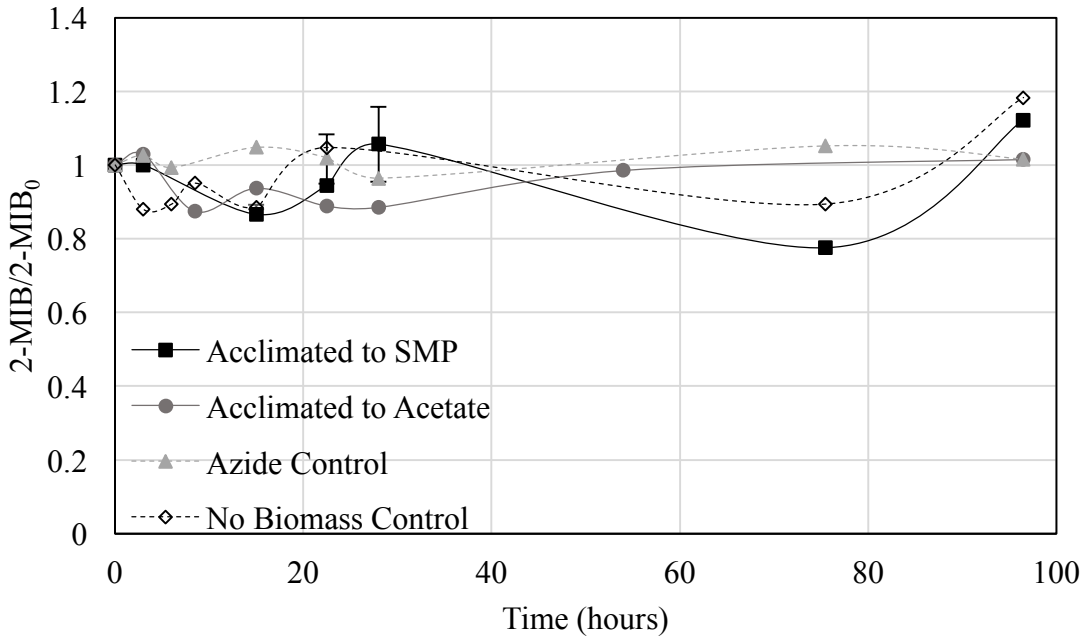

Figure 4.5. Fraction remaining of 2-MIB over time in *P. aeruginosa* culture. Initial conditions: 930 ng/L 2-MIB, 1.5 x 10^7 CFU/mL of *P. aeruginosa* acclimated to SMP, and 2.6 x 10^7 CFU/mL of *P. aeruginosa* acclimated to acetate. Sodium azide added at 1 g/L. Error bars are maximum and minimum of duplicate samples.

The observed fluctuations were likely due in part to analytical variability, as duplicate samples at two time points demonstrated 6.5 – 13.4% differences. As expected, no substantial removal of 2-MIB occurred in the azide control and in the no-biomass control. Relative to these controls, no substantial biological removal of 2-MIB by *P. aeruginosa* was observed. This was unexpected given the previous documentation of *P. aeruginosa* using 2-MIB as its primary substrate (Egashira et al., 1992). Egashira et al. isolated several microorganisms from a drinking-water biofilter to test if the isolates were 2-MIB degraders. The isolates were grown on 2-MIB as their sole carbon source and those with greater than 15% removal after 24 hours were designated 2-MIB degraders. After a multitude of bacterial identification tests, *P. aeruginosa* was identified as one of the 2-MIB degraders. The environmental *P. aeruginosa* isolate from that study was most likely not the same strain that was used in this study (PAO1), potentially explaining the discrepancy in biological removal.

Geosmin concentrations also fluctuated in these experiments (Figure 4.6). These fluctuations were likely due in part to analytical variability, as duplicate samples at three time points demonstrated 3.0 – 14.9% differences. The azide control showed 10% loss in geosmin after 96 hours, which is within the aforementioned analytical variability or could be due to volatile losses. Similarly, the no-biomass control showed approximately 20% loss of geosmin after 96 hours, which could be explained by a combination of analytical variability and volatilization. Relative to these controls, no substantial biological removal of geosmin by *P. aeruginosa* was observed. These results were anticipated as there are no published studies that demonstrate that *P. aeruginosa* can utilize geosmin as its primary substrate.

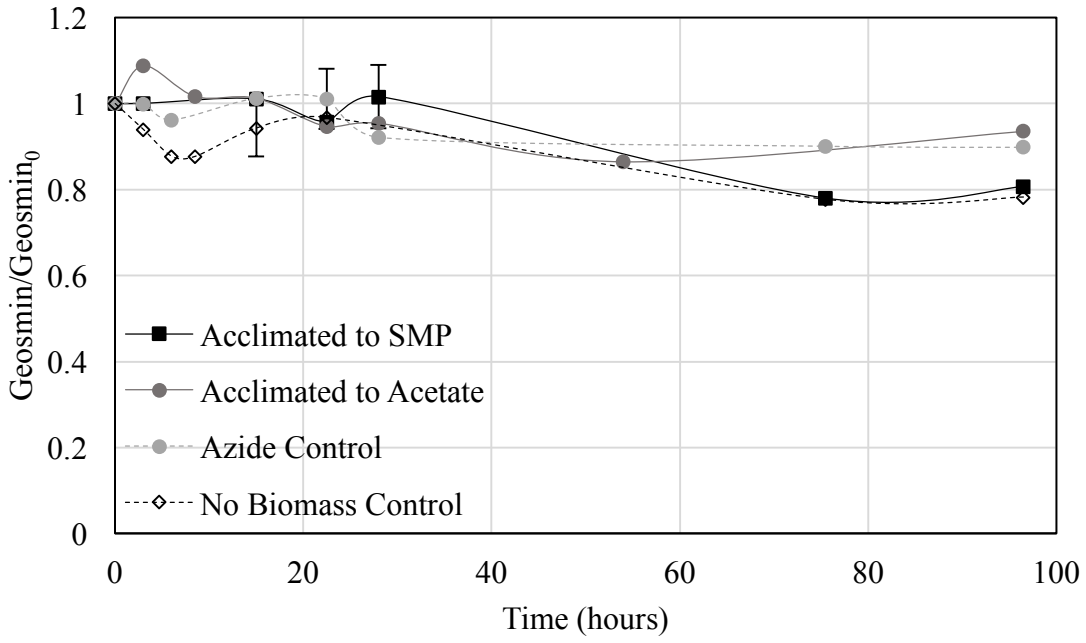

Figure 4.6. Fraction remaining of geosmin over time in *P. aeruginosa* culture. Initial conditions: 400 ng/L geosmin, 1.5 x 10^7 CFU/mL of *P. aeruginosa* acclimated to SMP, and 2.6 x 10^7 CFU/mL of *P. aeruginosa* acclimated to acetate. Sodium azide added at 1 g/L. Error bars are maximum and minimum of duplicate samples.

4.3.3 Removal of 2-MIB and Geosmin by Mixed Heterotrophic Culture

After acclimation to acetate or SMP, the washed and resuspended mixed heterotrophic communities were placed into synthetic water and spiked with 2-MIB and geosmin as the sole carbon and energy sources.

The azide control showed approximately 35% loss in 2-MIB after 96 hours (Figure 4.7), which is much higher than what was observed in the corresponding *P. aeruginosa* experiment (Figure 4.5); this suggests that azide might be a more effective inhibitor of *P. aeruginosa* than for the mixed heterotrophic community from AS. Thus, a fraction of the 35% loss in 2-MIB in the azide control for the mixed heterotrophic culture experiments is likely due to residual biological activity. The losses in 2-MIB for the mixed microbial communities acclimated to SMP or acetate were not substantially different from the azide control nor from each other. The differences are within the analytical variability observed for duplicate 2-MIB measurements (7.2 – 17.7%). These data suggest no advantage in 2-MIB biodegradation due to heterotrophic acclimation to this particular SMP as compared to acclimation to acetate.

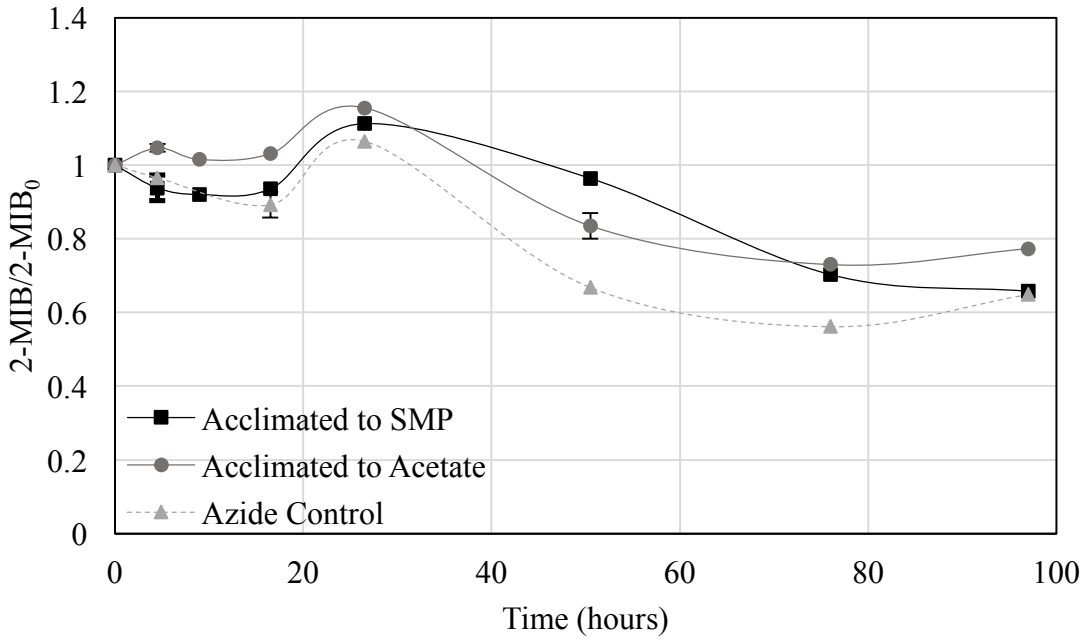

Figure 4.7. Fraction remaining of 2-MIB over time in mixed heterotrophic culture. Initial conditions: 530 ng/L 2-MIB, 1.4 x 10^6 CFU/mL of cells acclimated to SMP, and 4.2 x 10^6 CFU/mL of cells acclimated to acetate. Sodium azide added at 1 g/L. Error bars are maximum and minimum of duplicate analysis.

Similar to the 2-MIB results, the azide control showed approximately 35% loss in geosmin after 96 hours (Figure 4.8), which is much higher than what was observed in the corresponding *P. aeruginosa* experiment (Figure 4.6); again, this suggests that azide was a more effective inhibitor of *P. aeruginosa* than it was for the mixed heterotrophic culture from AS. Thus, a fraction of the 35% loss in geosmin in the azide control for the mixed heterotrophic culture experiments is likely due to residual biological activity. Although the fraction of geosmin remaining in the SMP-acclimated experiment was generally less than that remaining in the azide control and in the acetate-acclimated experiments, those differences are within the analytical variability observed for duplicate geosmin measurements (0.5 – 11.6%). These data suggest no substantial advantage in geosmin biodegradation due to heterotrophic acclimation to this particular SMP as compared to acclimation to acetate.

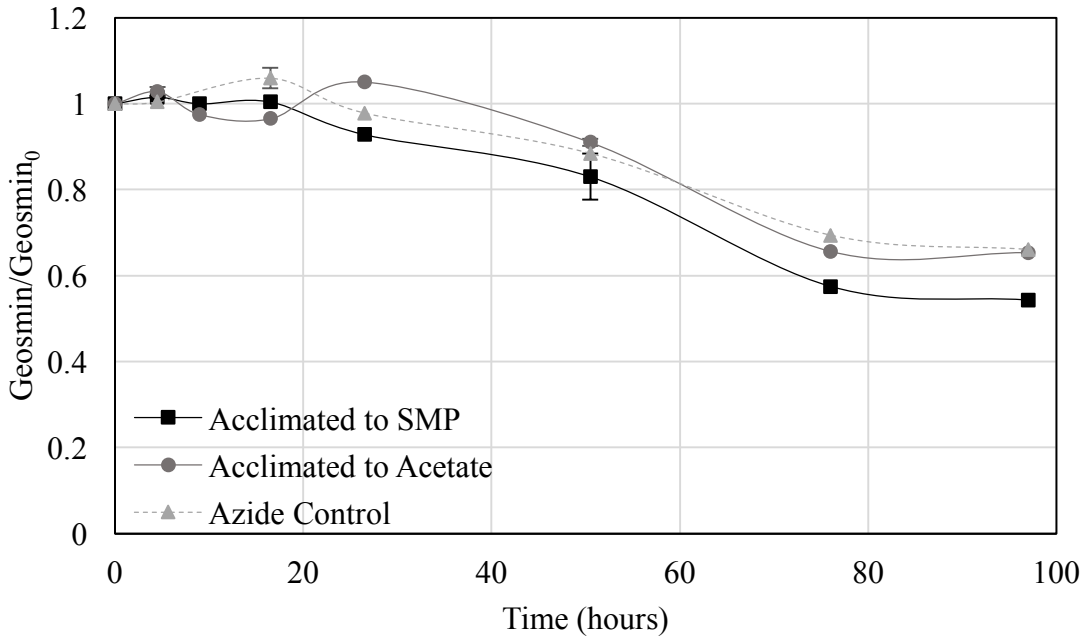

Figure 4.8. Fraction remaining of geosmin over time in mixed heterotrophic culture. Initial conditions: 280 ng/L geosmin, 1.4 x 10^6 CFU/mL of cells acclimated to SMP, and 4.2 x 10^6 CFU/mL of cells acclimated to acetate. Sodium azide added at 1 g/L. Error bars are maximum and minimum of duplicate analysis.

Acclimating *P. aeruginosa* and the mixed heterotrophic culture to the SMP obtained in this study did not appear to provide a substantial advantage to the subsequent biodegradation of the TrOC, 2-MIB and geosmin. The SMP used in this study were obtained from planktonic *N. europaea* cells, as opposed to a mixed culture of nitrifying bacteria in a biofilm. It has previously been shown that environmental conditions affect SMP characteristics (Barker et al., 2000; Barker & Stuckey, 2001). The structure, molecular weight distribution, and aromaticity of SMP in a drinking-water nitrification biofilter might be substantially different from those associated with the SMP utilized in this research. Thus, the results of this study might not reflect the effect that SMP have on heterotrophic metabolism and heterotrophs' ability to metabolize TrOC in a natural system.

4.3.4 Effect of Primary Substrate on Removal of 2-MIB and Geosmin by Mixed Heterotrophic Culture

After testing the heterotrophs with 2-MIB and geosmin as their sole carbon and energy source (4.3.3), SMP and acetate were spiked into the SMP-acclimated and acetate-acclimated flasks, respectively, containing the mixed heterotrophic community; no carbon source was spiked into the azide control. These spikes were to assess if the addition of a primary substrate would stimulate co-metabolic TrOC removal. For most experiments, the addition of SMP and acetate had minimal impact on the removal of 2-MIB and geosmin (Figure 4.9). However, for the experiment where SMP were supplemented to the heterotrophs, geosmin removal was substantially increased as compared to the azide control. At 80.5 hours after SMP addition, the fraction of geosmin remaining was 0.7 in the active heterotrophic experiment but 0.94 in the azide control. These results suggest SMP might act as a primary substrate for the co-metabolic removal of geosmin in a mixed heterotrophic culture.

These results are in agreement with a previous study examining the removal mechanisms of geosmin. Saito et al. (1999) showed that geosmin was not removed in drinking-water biofilters until the addition of a primary substrate. However, the necessity of a complex organic to act as the primary substrate had not been demonstrated previously.

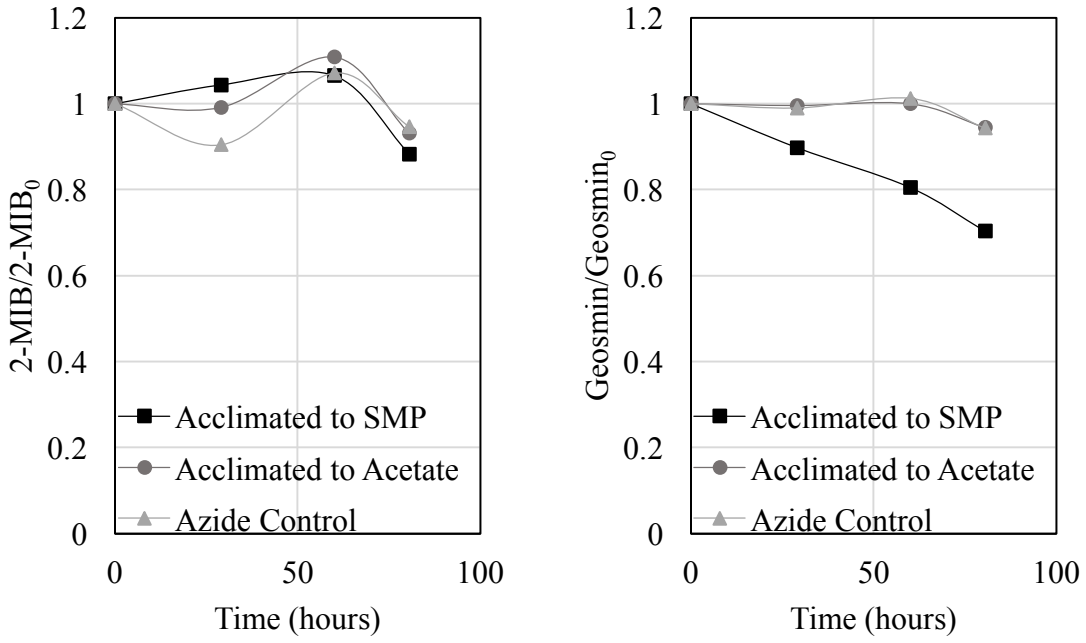

Figure 4.9. Effect of primary substrate (SMP or acetate) addition on fraction remaining of 2-MIB and geosmin by mixed heterotrophic community. Time 0 in these plots correspond to 117 hours after initial 2-MIB and geosmin spike in Figures 4.7 and 4.8. SMP or acetate were added at time 0 in these plots at 20 mg/L soluble COD, such that co-metabolism of the TrOC was examined.

CHAPTER 5: CONCLUSIONS

This chapter outlines the findings of this research along with recommendations for future work.

5.1 Summary of Findings

This study investigated whether heterotrophic microorganisms acclimated to a carbon source mixture, namely soluble microbial products (SMP), transformed trace organic contaminants (TrOC) at an increased rate as compared to heterotrophic microorganisms acclimated to a single, simple carbon source (acetate). SMP was produced by growing a dense culture of *Nitrosomonas europaea* for approximately 10 days and subsequently filtering out the biomass so only SMP remained. Batch experiments were conducted by feeding either a pure culture*, Pseudomonas aeruginosa*, or a mixed heterotrophic community with the simple organic compound acetate or SMP. After these microorganisms were acclimated to their respective carbon sources for 3-7 days, they were spiked with two TrOC, 2-methylisoborneol (2-MIB) and geosmin. In addition, there were two controls: heterotrophs amended with sodium azide to control for sorption to biomass, and a no-biomass control to account for sorption to glassware or volatilization. The removals of 2-MIB and geosmin in the cultures were examined over time. The main findings of this research are summarized as follows:

- The SMP produced had a total organic carbon concentration of 7.5 mg/L.
- A single freeze (-20°C)-thaw cycle of SMP did not change its ultraviolet (UV) absorbance at 254 nm.
- The SMP were in a high alkalinity solution due to the *N. europaea* growth method within a batch bioreactor. As *N. europaea* oxidized ammonium (thereby releasing protons), Na_2CO_3 was pumped into the bioreactor to maintain the pH at 7.8 and supply *N. europaea* with inorganic carbon for growth. The final alkalinity of the SMP solution was 2,360 mg/L as $CaCO_3$.

- The specific ultraviolet absorbance (SUVA) of the SMP was very low (0.083 mg/L-m), indicating that the SMP compounds in this study are generally low in molecular weight, aliphatic, and hydrophilic.
- Measuring the protein content of *P. aeruginosa* and the mixed heterotrophic culture was a good proxy for biomass.
- When supplied 2-MIB or geosmin as its sole carbon and energy source, *P. aeruginosa* did not biodegrade either within 96 hours under the tested conditions. Based on the literature, this result was expected for geosmin but not for 2-MIB.
- When supplied 2-MIB or geosmin as its sole carbon and energy source, the mixed heterotrophic community yielded approximately 35% removal of both TrOC, even when sodium azide was added to inhibit microbial activity. It is likely that azide provided insufficient control of microbial activity in the mixed heterotrophic community.
- The mixed heterotrophic community gained no substantial advantage in 2-MIB or geosmin biodegradation due to its acclimation to this particular SMP as compared to its acclimation to acetate.
- When SMP was added to the mixed heterotrophic culture in the presence of 2-MIB and geosmin, increased geosmin removal was observed as compared to the azide control. This suggests that SMP might instigate the co-metabolism of geosmin. Based on the literature, the necessity of a complex organic to act as the primary substrate had not been demonstrated previously.

5.2 Future Work

There are several paths forward to more fully determine the impact that SMP have on TrOC removal in drinking-water biofilters. This section outlines several possibilities for future work.

5.2.1 Collecting SMP from an Alternate Nitrifier Source

The SMP produced in this study were obtained from a planktonic culture of *N. europaea*; therefore, they might not be representative of the SMP found in a mixed nitrifier biofilm in a drinking-water biofilter. To address this, SMP should be produced from a mixed nitrifying biofilm. Furthermore, the SMP collection protocol utilized in this study could be improved by lysing the *N. europaea* cells at the end of batch growth period to increase biomass-associated products (BAP) in the SMP. A higher BAP fraction of the SMP would lead to more aromatic and higher molecular weight compounds (i.e., more complex compounds).

5.2.2 Inoculating with a Dense Inoculum

The biomass concentrations of *P. aeruginosa* and heterotrophs used in the 2-MIB and geosmin removal studies were relatively low. Use of higher biomass concentrations might produce more substantial changes in 2-MIB or geosmin concentrations within a reasonable timeframe, such that these concentration changes would exceed the analytical variability of the method. For instance, in this study, the heterotrophic culture acclimated to SMP removed geosmin to a slightly greater extent as compared to the heterotrophic culture acclimated to acetate (Figure 4.8). However, since the difference in geosmin removal between the two cultures was within the range of analytical variability; a definitive conclusion could not be drawn.

5.2.3 Natural Organic Matter as a Carbon Source

Only one source of SMP was used in the current study, and this SMP had a low SUVA value. To truly test the hypothesis that nitrifier SMP increases the rate of TrOC removal, future work should test multiple types of complex carbon sources, such as natural organic matter (NOM) in raw drinking water sources. Both NOM and SMP would be present in a nitrifying system; therefore, testing both carbon sources would be more representative of most practical nitrification applications. The SUVA value for NOM can

range from <2 mg/L-m to 6 mg/L-m, encompassing a diverse array of organic compounds (White et al., 1997). Several NOM are well characterized, such as the NOM in Suwannee River, Georgia (Averett et al., 1994). A comparison of NOM, SMP, or a combination of both as a carbon source for heterotrophs would provide more insight into the effect of a complex carbon source on TrOC removal in a nitrifying system.

5.2.4 Characterization of the SMP

Unknown in this research were the size distribution of the SMP and the concentration of assimilable organic carbon (AOC) of the SMP. Other studies have size-fractionated SMP (Barker & Stuckey, 1999), though not specifically for nitrifier SMP. Understanding the size distribution of SMP would provide evidence that the SMP is a complex mixture of organic compounds. Additionally, AOC is a standardized measurement of the heterotrophic bacterial growth potential in drinking water (van der Kooij, 1992) and would be more descriptive of the biodegradability of the SMP (as compared to total organic carbon or chemical oxygen demand measurements). Normalizing the AOC between the simple and complex carbon sources in the experiments would be a more accurate comparison.

APPENDIX

A.1. GC-MS: 2-MIB and Geosmin

Protocol for solid-phase microextraction (SPME) headspace analysis of 2-MIB and geosmin on Agilent GCMS 5977A

A.1.1 Supplies

- Analytical standard of 2-MIB + geosmin: 100 mg/mL in 1 mL of methanol, Sigma Aldrich CRM47525
- 2-Methylisoborneol, Sigma Aldrich 743364-5MG
- Geosmin, Sigma Aldrich UC18-5MG
- Internal standard of (±)-Geosmin ≥97% (GC), 200 mg/L in 5 mL of methanol, Sigma Aldrich 17932-1MG
- GCMS autosampler vials: CrossLab vial, screw top, headspace, 20 mL, amber, round bottom, 8010-0044
- GCMS additional caps to go with autosampler vials: CrossLab cap, magnetic, headspace, 18 mm, PTFE/silicone septum, 8010-0139
- SPME fiber: PDMS/DVB 65 µm, Sigma Aldrich 57327-U
- GCMS column, Agilent DB-5MS 122-5532UI, Ultra Inert, 30 m, 0.25 mm, 0.25 µm, 7-inch cage
- Molded Thermogreen LB-2 Septa, with injection hole diam. 11 mm, Sigma-Aldrich 28336-U
- SPME GCMS liner, Sigma-Aldrich 2637505
- Column nut for MS interface, part number 05988-20066
- O-ring, inlet liner, non-stick, 10/pk, part number 5188-5365
- Ferrule, 0.4 mm id, 15% graphite/85% Vespel, 0.1 to 0.25 mm column, short, 10/pk, part number 5181-3323
- Column nut for GC capillaries, 2/pk, part number 5181-8830

A.1.2 Preparation

- Clean glassware in Citrajet followed by 10% nitric acid bath
- Clean PTFE in Citrajet followed by 1% HCl bath
- Between baths clean in distilled water
- Leave glassware for at least 2 hours in each cleaning step
- Use only Nanopure water for stock solutions and standards
- Store stock solutions in the refrigerator
- All solutions at or above 10 ng/L should be handled in the fume hood due to the strong smell of 2-MIB and geosmin

A.1.3 Stock Solutions

- Add water to all vials according to Table A.1
- Add original stock solution
- Shake well and then continue with serial dilutions

Table A.1. Dilution of 2-MIB/geosmin stock solution.

Stock Solution	Units	Original Stock Solution	Units	Volume Stock (mL)	Volume DI Water (mL)
10	mg/L	100	mg/L	1	9
1	mg/L	10	mg/L	1	9
100	µg/L	1	mg/L	1	9
10	µg/L	100	µg/L	1	9
1	µg/L	10	µg/L	3	27

A.1.4 Internal Standard

- Clean syringe by flushing methanol first followed with Nanopure water
- Add 0.9 mL of Nanopure water to three 2-mL Agilent amber glass bottles
- Add the internal standard
- Shake well and continue with serial dilutions

Table A.2. Dilution of internal standard.

Internal Standard	Units	Original Stock Solution	Units	Volume Stock (µL)	Volume DI Water (µL)
20	mg/L	200	mg/L	100	900
2	mg/L	20	mg/L	100	900
200	µg/L	2	mg/L	100	900

A.1.5 Standard Curve

- Prior to creating standards add 3 g NaCl to each Agilent 15-mL amber glass bottle
- Add water to all vials according to Table A.3
- Add the stock solutions
- Add 5 µL of internal standard
- Immediately close lid

Table A.3. 2-MIB/geosmin standard curve preparation.

Stock Solution	Units	Original Stock Solution	Units	Volume Stock (mL)	Volume DI Water (mL)
1000	ng/L	10	µg/L	1	9
500	ng/L	1	µg/L	5	5
300	ng/L	1	µg/L	3	7
100	ng/L	1	µg/L	1	9
50	ng/L	1	µg/L	0.5	0.5
20	ng/L	1	µg/L	0.2	9.8

A.1.6 Prepare Auto Sampler, Syringe, and Fiber:

1. Set the agitator temperature: Gerstel → MPS Set Standby Temperature:
    a. Select Heater: Agitator
    b. Standby temperature: 80°C
    c. Click "Apply" and then "Close"
2. Set the tray: Gerstel → MPS Change Tray Types → Click "Yes"
    a. Select under 1: "Tray2"
    b. Select under 2: "VT32-20"
3. Set syringe: Gerstel → MPS Change Syringe → Click "Yes"
    a. Put the fiber into the syringe and place in the holder
    b. Select "Fiber" and then "OK"
    c. Bake syringe at 250°C for 60 minutes: Gerstel → MPS fiber bake
        i. Backout: Front
        ii. Bakeout time: 60
        iii. Bakeout Penetration (mm): 43
        iv. Click on "Close"

A.1.7 Load GC-MS Method

1. Method → Load Method→ Select Method Folder → Select ".M" method folder
2. GC Program: Instruments → GC Parameters
    a. Under the "Inlet" tab change the following:
        i. Heater: 250°C
        ii. Pressure: 7.6522
        iii. Septum purge flow: 3 mL/min
        iv. Septum purge flow mode: standard
        v. Splitless flow
        vi. Gas Saver: on
    b. Under the "Oven" tab input the following:

Rate	Value (°C)	Hold Time (min)	Run Time (min)
	50	1	
6	160	0	
20	300	5	31.33

3. MS program: Instruments → MS Edit Parameters
    a. Input the following:
        i. Acquisition type: SIM
        ii. 2-MIB: target 3 ions with m/z: 95, 107, 135; dwell time: 40 sec
        iii. Geosmin: target 4 ions with m/z: 97, 112, 115, 125; dwell time: 40 sec
        iv. Gain: 4
        v. Solvent delay: 11 minutes

4. Gerstel Autosampler Program: Gerstel → Edit Gerstel
    a. Input the following:
        i. Agitator temperature: 80°C
        ii. Agitator incubation: 2 minutes at 500 rpm
        iii. Extraction time: 10 minutes
        iv. Desorption: 5 minutes
        v. Post desorption bake out: 0 minutes if sample only contains 2-MIB and geosmin; 30 minutes if sample contains other TrOC

A.1.8 Tune the MS

- Instrument → Tune MSD, and then select "Tune MSD-ATUNE U"

A.1.9 Autosampler Program Preparation

1. Gerstel Prep → Load Prep Sequence → Select Prep Sequence Folder → Select folder with ".prep" file
2. Gerstel → Edit prep sequence
3. Pertinent information:
    a) Vial range: input the number of standards + samples
    b) For Source and Destination: Auto

A.1.10 Run Sequence

- Gerstel Prep → Run prep sequence → Select your Data File Directory → Run Sequence

A.1.11 Extracting Data

- Open program GC-MS Data Analysis
- Load method: Method → Load Method → Select Method

A.1.12 Standard Curves

Table A.4. Example standard curve responses.

Concentration	Unit	2-MIB	Geosmin	Internal Standard
20	ng/L	50396	129024	3949337
50	ng/L	119856	363341	3687755
100	ng/L	248099	855042	3401349
300	ng/L	749586	2389698	3878869
500	ng/L	1093423	3691810	3620990
1000	ng/L	2041578	7274662	3812151

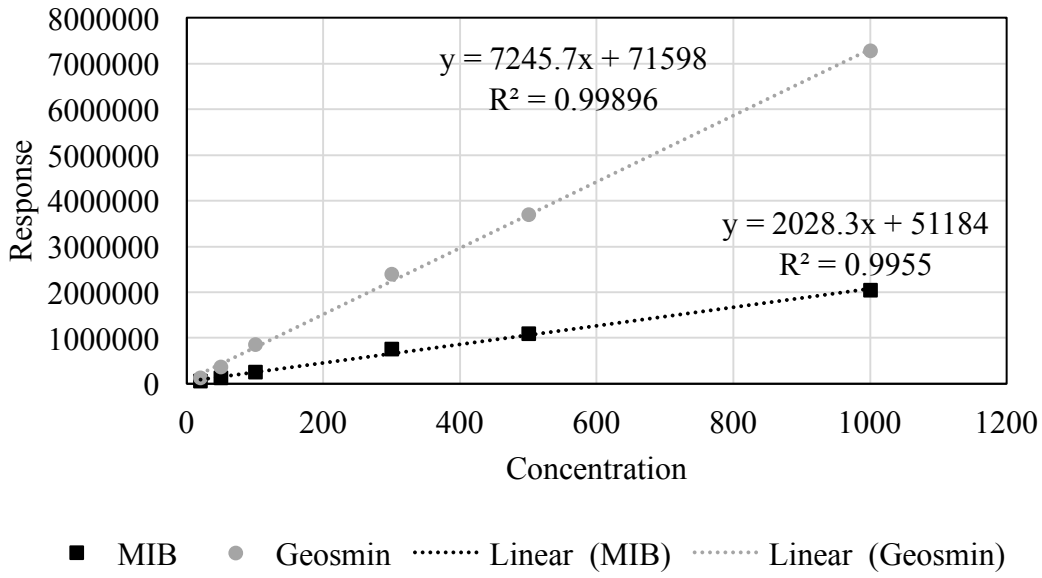

Figure A.1. Example standard curve for 2-MIB and geosmin.

- Normalize standard curves by calculating the relative response (RR) and relative concentration (Equation A.1 and Equation A.2)

$$RR = \frac{Response\ at\ Concentration\ X}{Response\ of\ Internal\ Standard\ at\ Concentration\ Y} \qquad \textbf{Equation A.1}$$

$$RC = \frac{Concentration\ of\ Standard\ X}{Concentration\ of\ Internal\ Standard\ Y} \qquad \textbf{Equation A.2}$$

- Plot the relative response versus relative concentration

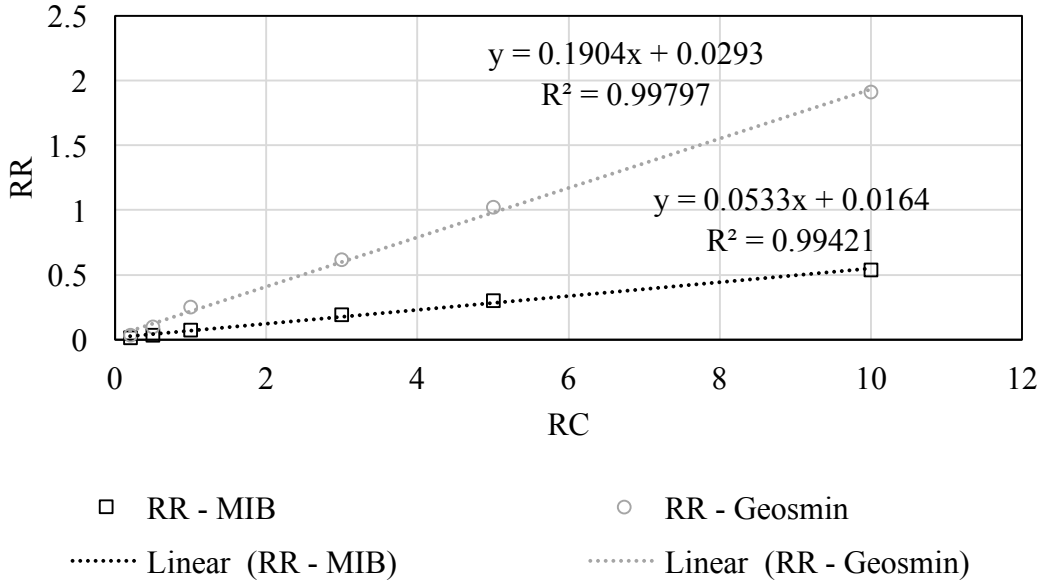

Figure A.2. Relative response versus relative concentration for 2-MIB and geosmin.

- The slope (m) and y-intercept (b) are used to calculate the concentration of a given sample with an internal standard (Equation A.3)

$$C_{Sample} = C_{Standard}\left[\frac{\left(\frac{Response\ of\ Sample}{Response\ of\ Internal\ Standard}\right)-b}{m}\right]$$

Equation A.3

A.1.13 Notes

- GCMS caps are 1-time use
- Fibers will last for at most 100 injections
- Septa should be changed after 50 injections
- Liner should be changed after 100 injections
- Between uses, the instrument method must be “Standby.M”

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